The mutagenesis enhancing activity of tumour promoting agents in cultured Chinese hamster cells.

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THE MUTAGENESIS ENHANCING ACTIVITY OF TUMOUR PROMOTING AGENTS IN CULTURED CHINESE HAMSTER CELLS

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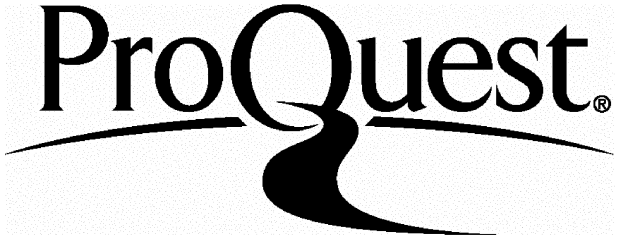
This research has been carried out in the School of Pharmacy and Pharmacology, University of Bath, under the supervision of C.J. Soper, B.Pharm., M.Sc., Ph.D., M.P.S.



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Paturient montes, nascetur ridiculus mus.

Horace

ABBREVIATIONS

AA - arachidonic acid
BHA - butylated hydroxyanisole
BHT - butylated hydroxytoluene
BZP - benzoyl peroxide
CA - chromosomal aberration(s)
CEP - 1(2-chloroethyl)piperidine
DMAE - dimethylaminoethyl chloride
DMAP - dimethylaminopropyl chloride
DMBA - 7,12-dimethylbenz[a]anthracene
DMN - dimethylnitrosamine
DMSO - dimethylsulphoxide
EGF - epidermal growth factor
EMEM - Eagle's minimal essential medium
EMEM+LC - Eagle's minimal essential medium supplemented with 2 x 10^{-4}M L-cysteine
EMS - ethylmethane sulphonate
Emt - emetine
EPP - ethylphenylpropiolate
F10 - Ham's F10 medium
F10(-HX) - Ham's F10 medium lacking hypoxanthine
FA - fluocinolone acetonide
HCHO - formaldehyde
HGPRT - hypoxanthine-guanine phosphoribosyl transferase (E.C. 2.4.2.8)
^{3}HPDBu - ^{3}H phorbol 12,13 dibutyrate
HX - hypoxanthine
LETS - large-external-transformation-sensitive protein
MAM - methylazoxymethanol
MGBG - methylglyoxal bis(guanylhydrazone)
MMS - methylmethane sulphonate
MNNG - N-methyl-N'-nitro-N-nitrosoguanidine
MNU - N-methyl-N-nitrosourea
MT - O^{6} methylguanine DNA methyl transferase (E.C. 2.1.1.66)
Na^{+}-K^{+}-Mg^{2+}-ATPase - sodium-potassium-magnesium-dependent adenosine triphosphatase (E.C. 3.6.1.3)
OAG - 1-oleoyl-2-acetylglycerol
ODC - ornithine decarboxylase (E.C. 4.1.1.17)
O^{6} MeG - O^{6} methylguanine
O^{4} MeT - O^{4} methylthymine
4-OMeTPA - 4-O methylTPA
Oua - ouabain
P_i - inorganic phosphate
PA - plasminogen activator
PDD - phorbol 12,13 didecanoate
PG - prostaglandin(s)
PGE - prostaglandin E
PGF - prostaglandin F
PKC - Ca^{2+}-phospholipid-dependent protein kinase C
PMN - polymorphonuclear leucocyte
Rb - rubidium
SAMD - S-adenosyl-L-methionine decarboxylase (E.C. 4.1.1.50)
SCE - sister chromatid exchange(s)
STS - skin tumour sensitive
TG - 6-thioguanine
TPA - 12-O-tetradecanoylphorbol-13-acetate
UDS - unscheduled DNA synthesis
UV - ultraviolet light

ABBREVIATIONS - CELL LINES

3T3 - established Swiss mouse fibroblasts
Balb/c 3T3 - mouse fibroblasts
C3H/10T½ - established mouse fibroblasts
CEF - chick embryo fibroblasts
CHO - established Chinese hamster ovary cell line(s)
CHO-K1 - subclone(s) of CHO
HEF - hamster embryo fibroblasts
HL-60 - human promyelocytic leukaemia cells
HTC - established rat hepatoma cell line
L5178Y - established mouse lymphoma cell line
MDCK - established dog kidney cell line
SHE - Syrian hamster embryo cells
V79 - established Chinese hamster lung fibroblasts (embryonic)
WI-38 - diploid human fibroblasts

SUMMARY

The introduction to this thesis reviews examples and characteristics of two-stage carcinogenesis, with particular reference to the promotion of mouse skin tumours by 12-O-tetradecanoylphorbol- 13-acetate (TPA). The effects of TPA and other tumour promoters on mammalian cells in culture is also discussed together with current evidence for the existence of specific phorbol diester receptors on the membranes of target cells. Chapter 2 lists the materials and methods basic to the cell culture procedures used in succeeding chapters.

The experimental work is presented in four parts. In Chapter 3, the effect of TPA on the frequency of both spontaneous and chemical-induced drug-resistant mutants of the Chinese hamster ovary cell line, CHO-K1, is described. The mutagen-specificity of the mutagenesis enhancing activity of TPA at the ouabain resistance (Oua^R) locus in these cells is confirmed. Using this Oua^R/CHO-K1 system the hypothesis that tumour promoting agents may act as *in vitro* mutagenesis enhancing agents via a free radical mechanism is investigated in Chapter 4. The influence of free radical scavenging compounds present in cell culture medium on the mutagenesis enhancing activity of TPA, benzoyl peroxide, formaldehyde and linear alkanes is examined. In Chapter 5 the effect of TPA on induced Oua^R mutation in other Chinese hamster cellsin culture is described.

Chapter 6 comprises a preliminary investigation into the survival of CHO-K1 cells irradiated in suspension with monochromatic ultraviolet light (UV). With reference to its membrane activity the effect of TPA on cell survival following UV irradiation is also examined.

A summary of findings is presented at the end of each experimental chapter. These are discussed in Chapter 7 in the light of recent hypotheses for *in vitro* mutagenesis enhancing activity.

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Considering their possible importance in human carcinogenesis little attention has been given to the detection of tumour promoting agents among endogenous and synthetic chemicals. Primarily, this has been due to the expense, both in terms of expenditure and time, that would be required to test environmental chemicals in currently available *in vivo* models of two-stage carcinogenesis. There is, therefore, an urgent need for a rapid screening test for compounds possessing tumour promoting activity.

The initiation of two-stage carcinogenesis is generally accepted to be a mutation event. As the majority of carcinogens are also mutagens (McCann et al., 1975), an attractive possibility is that tumour promoters may act in part to enhance mutagenesis at the gene level. The demonstration of such a mutagenesis enhancing activity might provide the basis of a screening test.

Mammalian cell mutation assays have been adapted to assess the mutagenesis enhancing activity of the potent tumour promoting agent TPA, but the data obtained have been conflicting. TPA has been found to enhance both chemical- and UV-induced gene mutations in Chinese hamster V79 fibroblasts (Lankas et al., 1977, 1980; Trosko et al., 1977). In cultured Chinese hamster ovary (CHO) cells, it has been suggested that TPA may only enhance the expression of specific MNNG-induced lesions (Dewdney and Söper, 1984). Conversely, Thompson et al. (1980) and Kinsella (1981) have reported no enhancement of UV-induced mutagenesis by TPA in either CHO or V79 cells, respectively. That different results are observed in different cell lines, even from the same species, is not surprising as *in vivo* tumour promoting agents are known to be tissue as well as species

specific (Table 1.1). A lack of reproducibility between laboratories employing the same cell line, however, questions the applicability of this as a valid screening method.

The purpose of this study was to investigate the influence of various factors implicated to explain these differences, the mutagen used, the cell culture conditions and the cell lines employed. It was envisaged that the results obtained may provide not only an explanation for the discordant results but also an insight into the mechanism of mutagenesis enhancement by TPA, and other tumour promoting agents.

CHAPTER 1. INTRODUCTION

1.1 Two Stage Carcinogenesis

In 1944 Mottram described an experimental model of carcinogenesis that provided conclusive evidence that tumour induction could be divided into two distinct treatment stages. The discovery of this 'initiation-promotion' protocol of tumour induction led to the proposal of the two-stage theory of carcinogenesis (Friedewald and Rous, 1944; Berenblum and Shubik, 1947). In the first stage, initiation, exposure of mouse skin to a single subthreshold dose of a carcinogen is postulated to cause changes resulting in the formation of latent tumour cells. During the second stage, promotion, repeated treatment of these 'initiated' cells with a tumour enhancing agent allows the expression of a neoplastic phenotype, and proliferation to a visible tumour. Inherent in the theory, and distinguishing promotion from other forms of carcinogenesis is that neither treatment alone is sufficient for tumour induction.

In the first experiments initiation was produced by a single application of benz[a]pyrene and promotion by repeated treatments with croton oil, the seed oil of *Croton tiglium* L. Until recently this remained the primary experimental model for the investigation of tumour promotion, principally because croton oil is an extremely potent promoter of mouse skin tumours (Boutwell, 1964). The identification of the active principles of croton oil as 12,13 diesters of phorbol, and the development of methods for their synthesis, made it possible to study the effects of chemically pure promoters (Van Duuren, 1969; Hecker, 1971). The availability of this homologous series of compounds, with a full range of tumour promoting potency, has prompted studies *in vivo* and *in vitro* aimed at the identification of the critical target sites in promotion. The most abundant and most potent of the series,

12-O-tetradecanoylphorbol-13-acetate (TPA), has now superceded croton oil as the promoter of choice. The phorbol diesters remain the most potent mouse skin tumour promoters, although a wide variety of other compounds have also been shown to possess tumour-promoting activity.

The molecular mechanisms underlying initiation and promotion are, as yet, undefined. There are, however, unique and distinct properties associated with each process. The initiation stage of skin tumour induction requires only a single subthreshold application of a carcinogen and is presumed to be a rapid process (Diamond et al., 1980). It is accomplished with no apparent morphological changes (Boutwell, 1964; Van Duuren, 1969), and initiated cells show no autonomy of growth (Farber, 1984a, 1984b). Furthermore, initiation appears to be irreversible. The time between initiation and promotion can be extended for up to a year, with little or no reduction in tumour yield (Boutwell, 1964, 1974; Van Duuren, 1969). This, despite the continued division of the epidermal cells from which the tumours eventually develop (Boutwell, 1974). It is now accepted that initiation involves the induction of genetic changes (Slaga et al., 1978a, 1982b; Diamond et al., 1980; Slaga, 1983), followed by fixation of these mutation events (Armuth and Berenblum, 1982; Trosko and Chang, 1984), which explains the irreversibility of the process. An important characteristic of initiators, inherent in the irreversible nature of the process, is that their effects are additive (Boutwell, 1964). If applied in sufficiently high single or cumulative doses the majority of initiators are also complete carcinogens. This is encompassed in the two stage theory of carcinogenesis by assumimg that these compounds possess both initiating and promoting properties (Boutwell, 1964, 1974; Farber, 1984a). The efficiency of the experimental initiation-promotion model can be attributed to croton oil, and TPA, being much more potent promoters than the complete carcinogens,

which at the low doses used for initiation lose their promoting activities (Boutwell, 1976). This may be an oversimplification. It is now becoming apparent that the nature of the 'promoting' component in complete carcinogenesis may differ from that in TPA promotion (Scribner and Scribner, 1982; Verma, 1982).

Promotion in marked contrast to initiation, is a slow process requiring prolonged exposure to the promoting agent (Mottram, 1944; Boutwell, 1964). Promotion itself may also be a two stage process (Boutwell, 1964; Slaga et al., 1980; Furstenberger et al., 1981), the first involving the 'conversion' of initiated cells into dormant tumour cells, and the second 'propagation' of these cells into visible tumours by means of cellular proliferation. This subdivision was originally proposed after it was found that a point was reached, after prolonged promotion of mouse skin, when the promoter could be replaced by a non-specific proliferative stimulus, e.g. turpentine, and tumours would still develop (Boutwell, 1964). The finding that a single application of TPA, followed by repeated treatments with a non-promoting hyperplastic agent is sufficient for tumour development after initiation further supports a two-stage promotion process (Furstenberger et al., 1981).

There is evidence that at least some part of the promotion process is reversible. When the interval between promoter applications is increased beyond an optimal time the promoting effect is greatly reduced or eliminated, even if the total promoting dose is kept constant (Boutwell, 1964). Reversibility is most probably related to the propagation stage in promotion with the first application of TPA inducing the obligatory effect for promotion (Furstenberger and Marks, 1983).

Promoters have been shown to elicit occasional tumours when applied repeatedly to mouse skin (Boutwell, 1964), and in some strains

of mice TPA appears to be a weak carcinogen (Astrup and Iversen, 1983). This apparent carcinogenic activity can be accounted for by the occurrence of a background of initiated cells or precancerous lesions, as a result of the actions of environmental carcinogens and spontaneous mutations (Van Duuren, 1969; Goerttler et al., 1982). It has been suggested that this property of tumour promotion could be used to detect background levels of initiation (Boutwell et al., 1982). It has, however, been demonstrated experimentally that tumour promoters are not just weak carcinogens. When repeated doses of promoters are followed by a single dose of an initiator no tumours result (Berenblum and Haran, 1955; Roe, 1959).

Despite conclusive evidence for two stages in mouse skin tumorigenesis, the relevance of the initiation-promotion protocol to general carcinogenesis remained in doubt for a considerable time. Using the same treatment schedule on the skin of other species of rodents negative results were obtained (Shubik, 1950). However, it is now becoming apparent, provided an appropriate experimental protocol is employed (Goerttler et al., 1982) that similar stages of tumour induction can be demonstrated in other species and tissues (Table 1.1). Other models of two-stage carcinogenesis are currently under evaluation, including a growing number of *in vitro* transformation systems. These have been reviewed by Hecker et al. (1982). An important finding in these studies is that a potential tumour promoter in one system may have no promoting activity in another. For example, both phorbol (Hecker, 1981) and phenobarbitone (Grube et al., 1975) are inactive in mouse skin but are active in other models (see Table 1.1). This suggests that different mechanisms of promotion are operating in different tissues.

Table 1.1 Animal models of two-stage carcinogenesis other than mouse skin.

TISSUE	INITIATOR	METHOD OF APPLICATION	PROMOTER	METHOD OF APPLICATION	REF
Mouse lung and liver	DMN	Sc injection	Phorbol	Ip injection	1
	DMN	Sc injection	Saccharin	Diet	2
Rat liver	AAF	Diet	Phenobarbitone	Diet	3
	AAF	Diet	Steroid drugs	Diet	4
	AAF	Diet	BHT	Diet	3
Rat bladder	MNU	Instilled in bladder	Saccharin	Diet	5
	MNU		Cyclamate	Diet	5
Rat colon	MNNG	Intrarectal	Bile acids	Diet	6
Mouse forestomach	DMBA	Intragastric	TPA	Intragastric	7
Rat mammary gland	DMBA	Intragastric	High fat	Diet	8
	DMBA	Intragastric	Progesterone	Im injection	9
Mouse skin and other organs	DMBA	Transplacental	TPA	Sc injection	10

Abbreviations

Im, intramuscular Ip, intraperitoneal Sc, subcutaneous
AAF, 2-acetylaminofluorene BHT, butylated hydroxytoluene
DMBA, 7,12-dimethylbenzanthracene DMN, dimethylnitrosamine
MNNG, N-methyl-N'-nitro-N-nitrosoguanidine MNU, methylnitrosourea

References

1 Armuth and Berenblum, 1972
2 Theiss et al., 1980
3 Peraino et al., 1978
4 Yager and Yager, 1980
5 Hicks et al., 1978
6 Reddy et al., 1978
7 Goerttler et al., 1979
8 Wynder et al., 1978
9 Yoshida et al., 1980
10 Goerttler and Loehrke, 1977

The diversity of compounds shown to have promoting activity in mouse skin, and other tissues suggests that there may be similar stages in human carcinogenesis. Significantly, the experimental models in which promotion has been shown to occur represent the sites of major cancer mortality in man (Sivak, 1978). A growing number of epidemiological studies have indicated that 60-90% of human cancers are induced by factors present in the environment (Doll, 1977; Wynder and Gori, 1977; Doll and Peto, 1981). The risk to man is probably from multiple exposures to a wide variety of chemicals resulting in complex interactions between initiators, cocarcinogens and tumour promoters (Diamond et al., 1980; Hecker, 1984). What cannot be distinguished from epidemiological studies is whether these environmental factors represent true promotion events or other forms of cocarcinogenesis. For one cancer, human lung cancer, there is circumstantial evidence for the role of promoting agents. The rapid decline in lung cancer risk in ex-smokers, compared to the risk in people who continue to smoke, suggests that some part of the tumour induction process is reversible (Doll, 1978; Wynder et al., 1978), and could result from the activity of promoting elements in the tobacco smoke. The role of promoting agents in other human cancers is now suspected. Apart from tobacco use, other factors particularly implicated are dietary constituents (Day, 1982; Hicks, 1982) and alcohol consumption (Wynder et al., 1978).

As promotion may be important in the induction of human cancer, it is a process that warrants further investigation. An understanding of the mechanisms involved may prove useful in the provision of more rational approaches to both cancer treatment and cancer prevention.

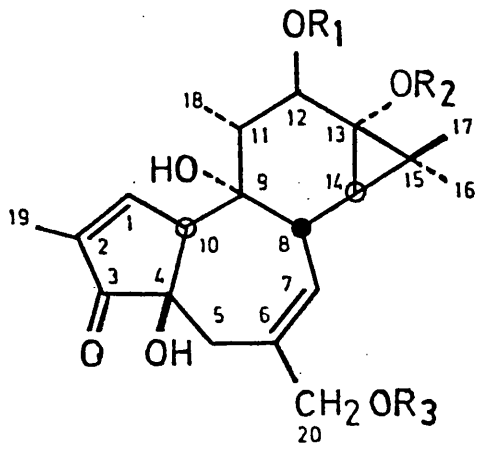
1.2 Chemistry of Tumour Promoting Agents

1.2.1 Structure-Activity Relationships

In biological processes where a number of agonists are available elucidation of their mechanism of action and their critical site of interaction has often been aided by the establishment of structure-activity relationships. For the tumour promoting agents such studies have been hampered not by a lack of agonists, but by the structural diversity of those available. The phorbol 12,13 diester promoting agents, however, provide a unique series of compounds for this type of study.

The general structure of these compounds was identified independently by Van Duuren (1969) and Hecker (1971) and is given in Fig. 1.1a. They are 12,13 diesters of the polyfunctional diterpene alcohol phorbol, with various aliphatic carboxylic acids.

Phorbol diesters are amphiphilic molecules consisting of a hydrophilic phorbol moiety, and a hydrophobic region, the saturated fatty acid chains at C-12 and C-13. The presence of the hydrophobic region appears to be essential for promoting activity in mouse skin, probably by assisting the passage of the molecule through a lipid environment to its site of action (Hecker, 1978). Phorbol, which lacks fatty acid side chains, is inactive as a mouse skin promoting agent (Hecker, 1971) except in the STS strain of mice (Baird and Boutwell, 1971). That the actual size of the hydrophobic region is important was shown by Thielmann and Hecker (1969), using a number of symmetrical 12,13 diesters. The promoting activity of these symmetrical diesters increased with increasing chain length up to 8 carbons but then, beginning with the 12-carbon diester the activity decreases markedly. For both symmetrical and asymmetrical diesters those having a combined carbon chain length of 14 to 20 are the most active (Hecker, 1978).



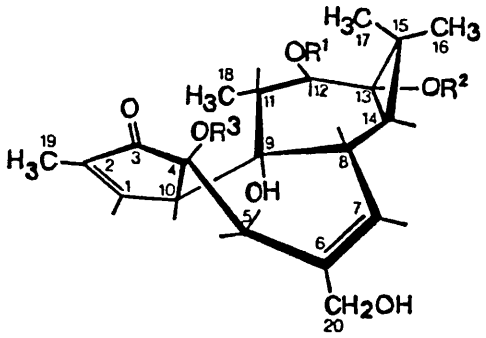
Phorbol R_1= R_2= R_3= H

TPA R_1= $C_{13}H_{27}CO$-

R_2= CH_3CO-

R_3= H

(a)



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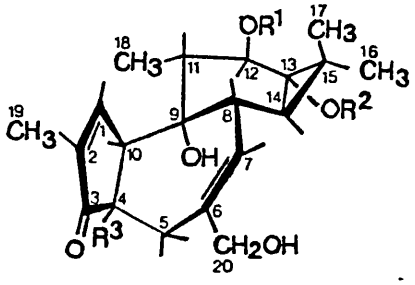

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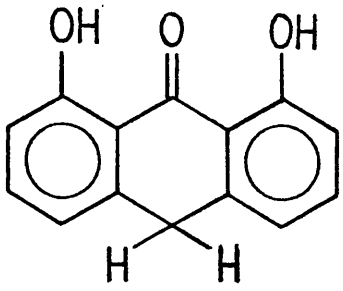


(d)

Fig. 1.1 Chemical structures of (a) Phorbol 12,13 diesters (b) 4β Phorbol 12,13 diesters (c) 4α Phorbol 12,13 diesters (d) Anthralin.

Phorbol diesters with aromatic side chains are less active than straight chain diesters containing the same numbers of carbons (Thielmann and Hecker, 1969).

The tumour promoting activity of the phorbol diesters is profoundly effected by alterations in the configuration of the phorbol moiety. In the active, 4β diesters, the 5- and 7-membered rings are trans-linked, Fig. 1.1b, conferring conformational rigidity on the molecule. On inversion of the C-4 hydroxyl group, producing the 4α diesters, Fig. 1.1.c, these rings become cis-linked. The molecule acquires flexibility, with a consequent loss of activity (Hecker, 1971). Loss of the C-4 hydroxyl group, however, does not significantly affect biological activity (Hecker, 1978). Methylation of the C-4 hydroxyl group leads to a great reduction in promoting activity, the TPA analogue 4-OMeTPA being only weakly promoting (Hecker, 1978). It has been suggested that 4-OMeTPA may be a useful negative control in promotion experiments (Hecker, 1978). However, it may not be the most appropriate compound for this use since it appears that its mechanism of action as a skin mitogen is different from that of TPA (Furstenberger et al., 1979, 1982a).

The presence of a free allylic hydroxyl group at C-20 is essential for high promoting activity (Hecker, 1968, 1971). Esterification of the 12,13 diesters at this position leads to production of 'cryptic' promoters, which require cleavage of the C-20 ester group to yield the active promoting species (Hecker, 1978).

The importance of the other functional groups on the phorbol moiety is less certain. It appears that the double bond at C1,C2, although not that at C6,C7, is required for skin tumour promoting activity. The cyclopropane ring system can also be opened without significant loss of activity (Hecker, 1978).

Limited studies on the structural requirements for biological activity have been performed for other tumour promoting agents. Diterpene ester promoting agents of the parent alcohols, resiniferonol and ingenol, exhibit similar structure activity requirements to those of the phorbol diesters (Evans and Soper, 1978; Fujiki et al., 1982a). All show the need for both hydrophobic and hydrophilic regions within the molecule. Furthermore, the triesters of ingenol appear to possess the cryptic promoting activity shown by the phorbol triesters (Fujika et al., 1982a).

For the non-diterpene ester-type promoting agents the structural requirements for activity are more diverse. The most potent of these compounds in routine use is the phenolic compound anthralin, Fig. 1.1d. Compared with TPA, it is much less active as a mouse skin promoter and needs to be applied in approximately 1000-fold higher doses to elicit a comparable tumour yield (Boutwell, 1974). For anthralin and its analogues the presence of the keto-phenol moiety is a pre-requisite for promoting activity (Van Duuren et al., 1978). For the phenol analogues shown to have weak promoting activity, no simple structural relationships could be determined, although over 50 compounds were studied (Boutwell and Bosch, 1959). Some of these phenolic compounds are thought to be responsible for the promoting activity of tobacco smoke condensate (Van Duuren et al., 1973). Carbon chain length is important for the linear alkane and alkanol promoting agents. Promoting activity for these resides solely in the compounds with 10-16 carbon chain length (Sice, 1966). While, the weak promoting activity of some long chain unsaturated fatty acids (Holsti, 1959), is increased in the methyl esters of the free acids (Arffmann and Glavind, 1971).

Considering the structural diversity of the mouse skin tumour promoting agents it seems impossible that any one mechanism will explain tumour promotion. On the basis of the potencies of the various tumour promoters it has been suggested that the mechanism of action of the phorbol diesters may be unique (Blumberg, 1981). The recent isolation of the indole alkaloids teleocidin from *Streptomyces* fungi (Fujiki et al., 1982b) and lyngbyatoxin A and debromoaplysiatoxin from blue-green algae (Fujiki et al., 1983) may confirm this. These compounds are chemically dissimilar from the phorbol diesters, yet appear to potentiate the same effects on mouse skin at similar doses (Sugimura et al., 1982).

1.2.2 Physical Properties of Phorbol Diesters

The physical properties of the phorbol diesters are a reflection of their amphiphilic nature. By measuring interfacial tensions of aqueous solutions of the diesters at 25°C Jacobson et al., (1975) obtained apparent solubility limits for TPA and phorbol didecanoate (PDD) of 2×10^{-6} M and 5×10^{-8} M respectively. This estimate for TPA is in close agreement with that of 3.7×10^{-6} M obtained by a spectrophotometric method (Van Duuren et al., 1976). As it is a surface-active agent, TPA also forms micelles. These have been detected in aqueous solution at 10^{-4} M (Edelman and Wang, 1978). The phorbol diesters interact with lipid structures *in vitro* (Jacobson et al., 1975). When introduced into the aqueous subphase, TPA, PDD or its inactive stereoisomer 4α PDD insert into phosphatidylcholine monolayers at the air-water interface, increasing surface pressure. Both TPA and 4α PDD also reduce the enthalpy of the minor transition of dipalmitoylphosphatidylcholine liposomes (Jacobson et al., 1975). This decrease was associated with a change in the orientation of the

acyl side chain of the phospholipid molecule. The ability to interact with phospholipid bilayers analogous to cellular membranes could be important to the tumour promoting activity of the phorbol diesters.

The phorbol esters are susceptible to autooxidation (Hecker, 1971). To prevent this Schmidt and Hecker (1975) have recommended that TPA, either as a solid or in solution should be stored at -20°C in the dark. Under these conditions these authors report no degradation of TPA for at least 6 months. In cell-free culture medium containing 10% foetal bovine serum both TPA and PDD have been found to be stable for at least 96 hours at 37°C (Berry et al., 1982).

1.2.3 Metabolism of Phorbol Diesters

As the phorbol diesters are capable of producing rapid effects both *in vivo* and *in vitro* (see below) it seems reasonable to expect that they do not require conversion to an active metabolite (Diamond et al., 1980; Blumberg, 1980). Experimental evidence has come to support this from studies both in mouse skin and in cell cultures.

Segal et al. (1975) failed to detect any metabolism of ^{3}HTPA during the first 24 hours after application to mouse skin. Similarly, Hecker (1978) found that up to 72 hours after administration of ^{3}HTPA to mouse skin the promoter molecule remained largely unchanged. In this study minor amounts of two metabolites, as opposed to autooxidation products (Schmidt and Hecker, 1975) were detected. Both were less active than TPA either as irritants or as promoters (Hecker, 1978). *In vivo* it is possible that some metabolites are not detected because of their rapid removal (Evans and Soper, 1978). This problem can be overcome by the use of *in vitro* systems, where metabolites accumulate in culture medium. In cell cultures TPA and other phorbol diesters are metabolised much more rapidly than *in vivo* (Diamond et

al., 1980). Different metabolites are produced by cells from different species (O'Brien and Diamond, 1978a; O'Brien and Saladik, 1980; Berry et al., 1982). In all cases the products of metabolism are of much lower activity than the original molecule. Information currently available indicates that any metabolism that does occur leads to inactivation rather than activation of TPA and other phorbol diesters.

1.3 Effects of Promoters on Mouse Skin

Mammalian skin consists of two distinct structures. The outer layer, the epidermis, is avascular and alymphatic, and serves to exclude harmful substances and micro-organisms. The inner layer, the dermis, is relatively acellular comprised largely of connective tissues with a few blood vessels. The dermis is responsible for the tensile strength of the skin. Malignant skin tumours are known to develop from epidermal cells (Boutwell, 1974). Consequently the majority of the studies on mouse skin have concentrated solely on the epidermis as the target for both initiators and promoters.

The application of phorbol diester tumour promoters to mouse skin leads to a number of alterations in epidermal morphology and metabolism (Raick, 1973a, 1973b). Similar changes have also been reported following the application of the non-phorbol promoting agents benzoyl peroxide (BZP) (Klein-Szanto and Slaga, 1982) and teleocidin (Fujika et al., 1982b). These changes appear to be the same whether or not the skin has been previously initiated (Argyris, 1983c).

The most conspicuous morphological events are an acute epidermal hyperplasia and cell proliferation (Raick, 1973a; Argyris, 1980). Associated with this stimulation of cell division are profound effects on macromolecular synthesis. A 0.016 µg application of TPA causes a sequential increase in tritiated precursor uptake into RNA, protein and, following an initial depression, DNA in both epidermis (Baird et al., 1971) or mouse whole skin (Raick, 1973a). The majority of these responses can also be elicited by other inflammatory hyperplasiogenic agents that are not promoting (Raick, 1974; Argyris, 1983b), although there may be some differences in the magnitude and onset of the responses (Raick, 1974; Nelson et al., 1982). Repeated applications of non-promoting hyperplastic agents are unable to maintain the diffuse

hyperplasia evident after repeated TPA treatments (Argyris, 1983a, 1983b). After a single application, promoters increase macromolecular synthesis above basal levels for 3-4 days. For the hyperplasiogenic agents this increase is not sustained longer than 24 hours (Baird et al., 1971). The induction of events associated with the preparation for cell division are not generally regarded as specific for promotion.

Tumour promotion in mouse skin, however, does seem inseparably linked to epidermal hyperproliferation. All the promoting agents so far studied have a strong mitogenic activity (Blumberg, 1981). For the phorbol diesters irritancy has been correlated with tumour promoting potency (Slaga et al., 1974). There are only a few exceptions, most notably, phorbol dibutyrate (Thielmann and Hecker, 1969) and the retinoyl derivative 12-O-retinoylphorbol-13-acetate (Furstenberger et al., 1981), which are both highly irritant but non-promoting. In addition, for TPA the threshold for the hyperplastic response is at least 1000-fold lower than that required for promotion (Boutwell, 1974). For the non-phorbol promoters, the association with hyperproliferation is not so clear, as many highly irritant compounds, such as acetic acid and turpentine, are at best only weakly promoting (Boutwell, 1964; Argyris, 1983b). Taken together,the weight of evidence seems to support the long held view that hyperplasia, though necessary for promotion, is not sufficient alone to completely explain the process (Gywnn and Salaman, 1953).

This complex set of tissue reactions evoked during the induction of hyperplasia creates a high background of changes in mouse skin. Thus, it has been difficult to determine the critical changes that distinguish the promoters from irritants in general. There are some changes, however, that cannot be simply ascribed to cell division or hyperplasia.

The induction of ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis, by promoters was first reported by O'Brien et al. (1975a). A 0.016 μg application of TPA stimulated ODC activity in mouse epidermis by more than 200-fold. The sharp peak of enzyme activity at 5-6 hours was followed by a return to low basal levels 12 hours after treatment (O'Brien et al., 1975a). Following this increase in ODC activity, epidermal levels of the polyamines, putrescine and spermidine, but not spermine, were elevated (O'Brien et al., 1976). The activity of another enzyme in polyamine biosynthesis, S-adenosyl-L-methionine decarboxylase (SAMD) was also increased by the application of tumour promoters to mouse skin (O'Brien et al., 1975a). This is not a specific event in promotion, as hyperplasiogenic agents also produce similar effects (O'Brien et al., 1975a).

The importance of ODC induction in promotion is suggested by other observations. Papillomas and carcinomas induced in mouse skin by the two stage protocol have elevated levels of ODC (O'Brien et al., 1976). Unlike many other regenerating systems, activation of this enzyme is not generally associated with cellular proliferation in mouse skin (O'Brien et al., 1975b). For TPA, the extent of ODC induction is dose-related (O'Brien et al., 1975b). In addition, and most significantly, for both phorbol and non-phorbol promoters the ability to induce ODC correlates well with skin tumour promoting activity (O'Brien et al., 1975b). The exception to this is the weakly promoting diterpene mezerein which has been found to induce ODC levels comparable to those induced by TPA (Mufson et al., 1979). However, as prior treatment of mouse skin with cycloheximide abolishes TPA induction of ODC (O'Brien et al., 1975a), *de novo* protein synthesis is

required for its expression. Activation of this enzyme, though necessary cannot therefore be the initial event in promotion.

A distinct morphological feature of promoter treated epidermis is the appearance of an increased number of keratinized cells, which reach a peak 4 days after the promoter treatment (Raick, 1973a; Klein-Szanto and Slaga, 1982). These 'dark' basal cells are distinguishable from normal epidermal cells by their smaller size and larger mitochondria (Evans and Soper, 1978). Non-promoting hyperplasiogenic agents such as ethylphenylpropiolate (EPP) seem unable to induce dark cells (Raick, 1974). The efficiency of dark cell induction correlates well with tumour promoting ability for both phorbol and non-phorbol promoters (Klein-Szanto and Slaga, 1981). The most potent mouse skin promoter TPA, increases dark cell numbers from 2-3% of basal cells to 15-20% (Klein-Szanto et al., 1982). The notable exception to this correlation is the weakly promoting 4-OMeTPA, which is an extremely good inducer of dark cells (Klein-Szanto and Slaga, 1981). A large number of these cells are found in papillomas and carcinomas induced in the two-stage protocol (Raick, 1974). Thus, dark cell induction has been considered the critical factor in the process of promotion (Bohrman, 1983). It has even been suggested that the failure of TPA to promote tumours in guinea pig skin is related to its inability to induce dark cells (Bourin et al., 1982). However, the relevance of dark cell induction to the promotion process remains uncertain.

There are some responses in mouse skin preceding those described above, which point to a membrane interaction as the initial event in promotion. Within 3 hours of 0.016 µg application of TPA the synthesis of phosphatidylcholine and phosphatidylethanolamine in mouse skin is markedly increased above control levels (Rohrschneider et al., 1972).

This 'early' stimulation of phospholipid metabolism by various phorbol esters correlates with their tumour promoting ability, and is not evoked by hyperplasiogenic agents (Rohrschneider et al., 1972). Significantly this prompt response is independent of protein synthesis (Rohrschneider and Boutwell, 1973a). Concurrent with this effect on phospholipid synthesis, TPA also causes an increased release of the prostaglandins, PGE and PGF, from cell membranes (Ashendel and Boutwell, 1979). This response has been reported to start within 15 minutes of promoter treatment (Furstenberger and Marks, 1980). Although PGs are known mediators of inflammation, their release may also be important to promotion. Pretreatment of mouse skin with indomethacin, an inhibitor of PG synthesis, abolishes promoter-induced ODC activity and DNA synthesis, as well as oedema (Verma et al., 1980). In addition, phorbol diesters have a known affinity for phospholipid bilayers and exhibit strict structural requirements for their biological activity, suggesting that they might interact with specific membrane sites. Promoter-membrane interactions could trigger conformational changes at receptor sites that may explain the reduced receptiveness of epidermal cells to endogenous growth inhibitors such as Gl chalone (Grimm and Marks, 1974) and β-adrenergic stimulation (Marks et al., 1978). Conformational changes may also be responsible for the selective activation of plasma membrane bound enzymes eventually leading to the nuclear events resulting in cell division (Rohrschneider et al., 1972). ODC induction by TPA is inhibited by colchicine, which implicates myotubules in the transmission of the signals from the cell membranes that effect gene expression (O'Brien et al., 1976).

It is possible that the targets for tumour promoters may not be confined to the epidermis. Little is known about promoter induced

effects in the dermis (Aldaz et al., 1985). The dermal changes that have been studied are those characteristic of inflammation. Increased vascular permeability, resulting in marked oedema, is seen within an hour of promoter application to mouse ear (Janoff et al., 1970). Accompanying these vascular changes, leucocytes have been shown to infiltrate the dermis within 24 hours (Frei and Stephens, 1968). This leucocyte infiltration may be important to promotion. When treated with TPA *in vitro* leucocytes are known to produce an 'inflammatory burst' of activity resulting in the release of proteolytic enzymes and reactive oxygen radicals (Goldstein et al., 1979; Witz et al., 1980). Interest in these reactive species has been increased with the finding that TPA and other promoting agents, but not hyperplasiogenic agents, decrease epidermal levels of superoxide dismutase (SOD) and catalase (Solanki et al., 1981). These enzymes are part of the major cellular defence mechanism against reactive oxygen species (Fridovich, 1974). As the dermis has an essential role in epidermal physiology, these changes should not be disregarded, and may play a significant, but as yet undefined, role in promotion (Aldaz et al., 1985).

1.4 Inhibition of Mouse Skin Tumour Promotion

Various modifiers of tumour promotion have been very useful in increasing the understanding of the cellular events critical to the process of promotion. The anti-inflammatory steroids, such as fluocinolone acetonide (FA), are the most potent inhibitors of mouse skin tumour promotion (Schwarz et al., 1977; Slaga et al, 1978b). The repeated application of as little as 0.01 μg of FA almost completely inhibits phorbol ester-mediated tumour promotion, cellular proliferation and dark cell induction in mouse skin (Schwarz et al., 1977; Slaga et al., 1980). FA does not, however, inhibit promoter induced ODC activity (Slaga et al, 1983). Certain retinoids are also potent inhibitors of phorbol diester tumour promotion (Verma et al., 1979). In contrast to FA, the active retinoids inhibited promoter-induced epidermal ODC activity, but are ineffective against the production of hyperplasia and dark cells (Verma et al., 1979). The application of a combination of FA and retinoids to mouse skin, concurrently with TPA, acts synergistically to completely inhibit tumour promotion (Weeks et al., 1979).

The differential spectrum of activity of these inhibitors supports the subdivision of promotion into two stages (Boutwell, 1964; Furstenberger et al., 1981) and indicates that different cellular events are critical to each stage. Dark cell induction is correlated with Stage I (conversion) activity, while ODC activation is correlated with Stage II (propagation) activity (Slaga et al., 1982). The association of different critical effects within these stages can be used to explain the anomaly of the weak promoters 4-OMeTPA and mezerein. 4-OMeTPA, a potent inducer of dark cells, is now classified as a Stage I promoting agent, requiring only a single application to accomplish the conversion of an initiated cell to a dormant tumour

cell (Furstenberger et al., 1982a). Mezerein, a potent stimulator of ODC activity is classified as a Stage II promoter. Multiple applications of mezerein are required following a single application of a Stage I promoter for tumours to become visible (Slaga et al., 1983).

The effects of other inhibitors, although less impressive, do indicate that other promoter-mediated effects may be of some importance. Various protease inhibitors, including the chloromethyl ketones, antipain and leupeptin, suppress mouse skin tumour promotion (Troll et al., 1978). As there is no clear evidence that tumour promoters stimulate protease activity in mouse skin, these agents may not be specifically inhibiting the action of proteases (Diamond et al., 1980). The prostaglandin synthesis inhibitor, indomethacin, is only a weak inhibitor at low doses (Viaje et al., 1977). However, this lack of inhibitory effect may be due to its low lipophilicity preventing percutaneous absorption (Slaga et al., 1978b). Other metabolites of arachidonic acid may be important in tumour promotion. Fischer et al. (1982) have shown that phospholipase A_2 inhibitors and thromboxane synthesis inhibitors also suppress TPA-mediated tumour promotion.

Recently, the antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been found to be potent inhibitors of skin tumour promotion by both TPA and BZP (Slaga et al., 1983). Their mechanism of action remains to be elucidated, but it is possible that free radicals are of importance and these agents may prevent promotion by their free radical scavenging potential.

1.5 Effects of Tumour Promoters on Cells in Culture

In the study of tumour promotion, cell culture systems offer the advantages of being better defined and more flexible than *in vivo* systems. As they are also simpler, the experimental results obtained are more easily interpreted. The effects of tumour promoters have been investigated on

a) primary cultures - cultures initiated directly from an organism;

b) primary cell lines - cell populations derived at the first or subsequent subcultures from primary cultures, which can only be passaged a finite number of times; and

c) established cell lines - which demonstrate the ability to be subcultured indefinitely.

All of these especially established cell lines, represent cells somewhat altered from the original cells *in vivo*. The extrapolation of results to the *in vivo* situation, consequently, must be undertaken with caution. However, the findings of *in vitro* studies are useful because they often reflect, and extend, the observations made in mouse skin.

The majority of *in vitro* studies have employed the phorbol diesters, in particular TPA. The use of other non-phorbol promoting agents has been restricted by their toxicity to cells in culture.

Many studies with tumour promoting agents have attempted to mimic two-stage carcinogenesis *in vitro*. These have usually employed established rodent cell lines, which undergo malignant transformation following exposure to oncogenic viruses and chemicals (Pienta et al., 1983). This is characterised by the loss of the contact-dependent inhibition of cell division, which normally limits their saturation density in monolayer culture, and the formation of piled-up criss-crossed foci (Paul, 1975; Heidelberger, 1976). Concurrently,

they undergo morphological changes and acquire the capacity to grow in soft agar and to give rise to tumours when injected into an appropriate animal host (Heidelberger, 1976).

In 1974 Lasne and coworkers described what appeared to be two-stage malignant transformation of cells in culture. They reported the acceleration of the time required for the transformation of rat embryo fibroblasts when they were treated with benzo[a]pyrene and subsequently grown in TPA-containing medium (Lasne et al., 1974). However, on further investigation transformation of this cell line was found to occur in most experimental groups, including those with carcinogen alone, TPA alone and untreated cells (Lasne et al., 1977).

Of the other transformation systems in which TPA is active the most convincing and best studied example of two-stage carcinogenesis *in vitro* employs the established C3H/10T½ mouse fibroblast cell line. In cultures of this cell line continuous exposure to a non-toxic, non-transforming concentration of TPA (1.6×10^{-7} M), following treatment with a subtransforming dose of either a carcinogenic hydrocarbon or ultraviolet light, produces a substantial number of transformed foci (Mondal et al., 1976; Mondal and Heidelberger, 1976). For transformation to occur the promoters must be given after the 'initiator', and for a series of phorbol diesters their potency in this system parallels their *in vivo* promoting activity (Mondal et al., 1976). Furthermore, a number of non-phorbol promoters have also been shown to promote transformation of this cell line (Heidelberger and Mondal, 1982; Hirakawa et al., 1982; Kaibara et al., 1984).

Another transformation system which may become useful to promoter research uses Syrian hamster embryo (SHE) fibroblasts (Poiley et al., 1979). With this cell line, in addition to transformation,

which can be demonstrated after only 7 days (Di Paolo et al., 1982), parallel mutagenesis studies can be performed at two genetic loci (Barrett et al., 1983).

Although the systems described above are operationally analogous to *in vivo* studies they all utilize cells of a fibroblastic origin, rather than epithelial cells from which the majority of tumours are known to develop. No clear cut model of two-stage transformation has yet been documented in epithelial cell cultures, despite extensive efforts. This is principally because of the technical difficulties involved in maintaining epidermal cells in culture (Bohrman, 1983; Yuspa et al., 1983).

In addition to the effects observed in transformation systems, promoting agents have also been shown to evoke pleiotropic effects in diverse cell types. It must be emphasised that these effects represent the responses of 'uninitiated' cells, and that effects observed in one particular cell type may not be common to others. However, when a series of phorbol diesters have been tested for a specific effect *in vitro* a positive correlation has generally been found between promoting activity in mouse skin and the ability to induce that effect *in vitro* (Diamond et al., 1980).

TPA can have striking reversible effects on the morphology and size of cells in monolayer culture. These effects are most apparent in sparse, growing cultures, although similar changes do occur in confluent cultures (Diamond et al., 1980).

Primary cultures of newborn mouse epidermis can be maintained in culture (Yuspa et al., 1976a; Fusenig and Samsel, 1978). These cells differentiate *in vitro* and produce keratin proteins that are identical to those of the outer horny layer of the skin. Addition of TPA (10^{-8} - 10^{-5} M) to these cells alters their morphology, shifting them to a

less differentiated state, with wider intercellular spaces (Yuspa et al., 1976a; Fusenig and Samsel, 1978); changes resembling those seen in mouse skin treated with TPA (Raick, 1973a). Similar morphological changes have also been reported in cultured human epidermal keratinocytes exposed to TPA (10^{-9} - 10^{-8} M) (Parkinson and Emmerson, 1982).

The treatment of primary fibroblast cultures with TPA also results in morphological changes. The cells of growing cultures of WI-38 diploid human fibroblasts (Diamond et al., 1974), or chick embryo fibroblasts (CEF) (Driedger and Blumberg, 1977) become elongated on exposure to low concentrations of TPA (1.6×10^{-7} M and 5×10^{-8} M, respectively). Concurrently, the number of cytoplasmic processes is increased, and the nuclear and cytoplasmic membranes show more distinctly than in control cultures. During exposure to TPA, WI-38 cells retain a normal well-orientated growth pattern (Diamond et al., 1974) while CEF assume a criss-crossed disorientated arrangement (Driedger and Blumberg, 1977). For CEF this change in growth pattern is similar to, but distinguishable from that induced by transformation with Rous sarcoma virus (RSV) (Wilson and Reich, 1979). In contrast to the phorbol diesters, non-phorbol promoting agents do not induce morphological changes in CEF (Driedger and Blumberg, 1978).

In established Swiss 3T3 mouse fibroblasts treated with 8×10^{-8} M TPA changes in cellular morphology are similar to those reported for WI-38 fibroblasts (Diamond et al., 1974). With continuous TPA exposure sparse cultures of WI-38, 3T3 or CEF all achieve higher saturation densities than control cultures (Diamond et al., 1974; Driedger and Blumberg, 1977). This is probably the result of a decrease in cell volume, associated with TPA treatment, which delays the onset of contact-dependent growth inhibition (Diamond et al., 1974).

The induction of epidermal macromolecular synthesis and cellular proliferation are characteristic actions of tumour promoters *in vivo*. TPA is capable of stimulating these events *in vitro*, particularly in cells which have become quiescent. It has been proposed that this is an effect mediated through the cell membrane (Jazwinski et al., 1978; Tsien et al., 1982), with TPA, or other phorbol diesters, directly stimulating cell replication mechanisms and/or interrupting endogenous growth controls (such as contact-inhibition).

When contact-inhibited cultures of Swiss 3T3 mouse fibroblasts were exposed to TPA (8×10^{-7} M) a sequential stimulation of RNA and DNA synthesis was produced (Sivak and Van Duuren, 1970). The amount of protein per culture also increased after 4 days continuous exposure to TPA. Similar observations have been made using confluent, contact-inhibited Balb/c3T3 mouse fibroblasts (Sivak, 1977) and human fibroblasts (O'Brien et al., 1979). In quiescent cultures of hamster embryo fibroblasts (HEF), however, DNA synthesis was not stimulated in response to phorbol diesters (O'Brien and Diamond, 1977).

DNA synthesis and cell proliferation can also be elevated by TPA in several growing cell culture sytems. In many cases, however, it appears that the observed effects on growth parameters may be a rebound phenomenon, due entirely to the synchronisation of cells during an initial inhibition of DNA synthesis by TPA (Peterson et al., 1977). However, this explanation may not be applicable to all cases. In primary cultures of newborn mouse epidermal cells, following an initial depression, TPA causes a pronounced and sustained stimulation of DNA synthesis (Fusenig and Samsel, 1978). As TPA also increases the proliferative compartment of the cell population this response is not merely an overshoot reaction following the preceding block (Fusenig and Samsel, 1978). The stimulation of DNA synthesis by TPA in this

system is dose-dependent over the range 10^{-9} - 10^{-5} M, and for a series of phorbol diesters there is a good correlation between the ability to stimulate DNA synthesis and tumour promoting ability (Yuspa et al., 1976b). The similarity of the results in this *in vitro* model with those *in vivo* (Raick, 1973a) suggests that the enhancement of epidermal cell growth by phorbol diesters is caused by a direct interaction with the target cells, and is not simply part of a non-specific inflammatory response.

A similar response is shown by growing C3H/10T½ cells to promoter treatment (Weinstein et al., 1978). TPA (4.8 x 10^{-7} M) causes an initial transient inhibition of exponential growth, which is followed by a return to the original growth rate, rather than a stimulation of growth. TPA-treated C3H/10T½ cultures do, however, go on to achieve a 4-fold increase over controls in saturation density (Weinstein et al., 1978); analogous to the promoter-induced hyperplasia in mouse skin.

A large number of studies have examined specific biochemical effects of phorbol diesters in cells in culture. As in adult mouse epidermis *in vivo* TPA increases ODC activity in primary newborn mouse epidermal cell cultures. TPA (1.6 x 10^{-7} M) induces a 5-fold increase in ODC-activity, with peak induction at 9 hr (Yuspa et al., 1976b). For TPA and five other phorbol diesters a rank-order correlation of ODC stimulation with promoting activity has been demonstrated (Yuspa et al., 1976b). O'Brien and Diamond (1978b) have shown that phorbol diester tumour promoters also induce ODC in HEF cell cultures, and that the extent of this induction was greater in cells which had undergone malignant transformation than in normal HEF. In contrast to mouse epidermal cells *in vitro* and *in vivo*, the induction of ODC by TPA in HEF is not followed by an increase in DNA synthesis. Enhanced

ODC activity is not, therefore, simply a prelude to promoter-induced cell proliferation (O'Brien and Diamond, 1978b).

An effect of tumour promoters of particular importance is the production of reversible changes in a number of specific cellular proteins normally associated with the transformed phenotype. The most extensively studied of these are the loss of the major cell surface protein, LETS (large-external-transformation-sensitive) protein, and the induction of plasminogen activator.

The function of the LETS protein is not known (Hynes, 1976), although its loss from the cell surface correlates well with malignant transformation in a wide variety of cell types (Blumberg, 1981). The treatment of exponentially growing cultures of CEF with TPA (1.6×10^{-8} M) leads to a decrease in the amount of LETS protein on the cell surface, with the total amount being decreased by 80% after 3 days (Blumberg et al., 1976). Within 3 days of subculture into medium without TPA the treated cells regain normal levels of LETS.

Plasminogen activators (PA), which act in the fibrinolytic system to convert plasminogen to plasmin, are detected in many tissues in the body. In most cell types *in vitro* malignant transformation is accompanied by a marked increase in PA levels (Blumberg, 1981) and *in vivo* increased PA secretion may be associated with the metastatic behaviour of tumour cells (Wang et al., 1980). The observation that TPA is a reversible inducer of PA in a number of cell types including CEF, HeLa cells and HTC rat hepatoma cells (Wigler and Weinstein, 1976) is therefore of importance. In CEF, PA is induced by concentrations of TPA as low as 5×10^{-9} M and requires *de novo* RNA and protein synthesis (Wigler and Weinstein, 1976). The effect is observed as early as 3 hr after exposure to TPA, and is sustained provided TPA remains in the medium. PA returns to normal levels within

12 hr of the removal of TPA. For a series of phorbol diesters, and related diterpenes, the induction of PA correlates with tumour-promoting potency (Weinstein et al., 1978).

The involvement of PA in tumour promotion has, however, been questioned. Several agents known to inhibit mouse skin tumour promotion, including retinoic acid and dexamethasone, fail to block the elevation of PA levels by TPA in CEF or HeLa cell cultures (Weinstein et al., 1978). Additionally, non-diterpene ester-type promoters do not induce PA in CEF (Weinstein et al., 1978).

As carcinogenesis is likely to involve major disturbance of cell differentiation (Sporn, 1978; Weinstein et al., 1979), the actions of the phorbol diesters on various programmes of cellular differentiation *in vitro* has drawn considerable attention (Tables 1.2a and 1.2b). At first sight, this dual effect of the tumour promoters appears confusing. However, the observations that the phorbol diesters inhibit terminal differentiation is in agreement with indications *in vivo* that TPA inhibits differentiation (keratinization) in mouse epidermis (Raick, 1973a; Marks et al., 1978). This action may be of importance with regard to the tumour-promoting activity of these compounds (Yamasaki et al., 1982). The induction of differentiation by the phorbol diesters in other cell culture systems (Table 1.2b) may partially explain why some of these compounds have an antileukaemic action (Evans and Soper, 1978; Hecker, 1978).

Studies on mouse skin suggest that the cell membrane is the major target for the action of the phorbol diesters (1.3). The effect of these compounds on isolated membrane preparations (Kubinski et al., 1973; Van Duuren et al., 1976) indicate that they may also be capable of disrupting membrane integrity. The effects of TPA on cell culture systems, described above, e.g. changes in cellular morphology and

Table 1.2 Examples of the modulation of cell differentiation *in vitro* by phorbol diesters.

CELL SYSTEM	TYPE OF DIFFERENTIATION	REFERENCE
a) Inhibition		
Cultured mouse epidermal cells	Keratinization	Fusenig and Samsel, 1978
Cultured hamster epidermal cells	Keratinization	Scribner and Suss, 1978
Friend murine erythroleukaemia	Erythroid	Yamasaki et al., 1977
Balb/c 3T3 mouse fibroblasts	Adipose conversion	Diamond et al., 1977
Chick embryo myoblasts	Myogenesis	Cohen et al., 1977
b) Induction		
Rauscher virus-transformed murine erythroleukaemia	Erythroid	Miao et al., 1978
Human T lymphoblasts	E-rosette formation	Nagasawa and Muk, 1980
Human promyelocytic leukaemia	Myeloid	Huberman and Callahan, 1979
Human melanoma	Melanogenesis	Huberman et al., 1979

orientation, loss of contact-inhibition of growth, and loss of LETS proteins, support this hypothesis. In addition, promoters induce changes in certain physical parameters, which are most probably due to direct effects on cell membranes. Wenner et al. (1974) observed that TPA (10^{-6} M) caused a decrease of 15% in the electrophoretic mobility of Erhlich-Lettre ascites tumour cells in culture, while the non-promoting 4α PDD did not. This decrease in mobility could be reversed by washing the cells. Also, the fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene, embedded in the cell membranes of lymphoblastoid and rat embryo cells, is decreased following treatment with promoters (Castagna et al., 1979; Fisher et al., 1979). As this decrease was not observed with non-promoters it has been proposed that in the presence of promoting agents the lipid moieties of cell membranes may become more fluid (Fisher et al., 1979).

A number of biochemical effects induced by phorbol diester tumour promoters on cells in culture are almost certainly the result of membrane modifications. The rapidity of onset of these changes, and the fact that they do not require *de novo* RNA and protein synthesis indicates that they are the direct result of exposure to phorbol diesters.

As in mouse epidermis *in vivo*, the phorbol diesters stimulate phospholipid synthesis in certain cell cultures. TPA treatment of HeLa cells (Suss et al., 1971) and bovine lymphocytes (Wertz and Mueller, 1978) increases the incorporation of ^{3}H-choline into their membrane phospholipids. In bovine lymphocytes, the ability of several phorbol diesters to stimulate ^{3}H-choline incorporation correlates with their potency as tumour promoters (Wertz and Mueller, 1978).

The effects of TPA on the membrane transport of low molecular weight compounds is dependent on the system examined. These effects

are of particular interest because they can be easily measured and are among the earliest effects observed following treatment with TPA. The uptake of $^{86}Rb^{+}$, a K^{+} analogue, and $^{32}P_i$ into quiescent Swiss 3T3 mouse fibroblasts in serum-free medium is increased within 5-10 min of the addition of TPA (5×10^{-8} M) (Moroney et al., 1978). In this cell line stimulation of uptake is ouabain-sensitive, suggesting that membrane-bound Na^{+}/K^{+}-ATPase is the target for phorbol diesters. The rate of transport of a glucose analogue, 2-deoxyglucose, is also increased in several cell types (Blumberg, 1980). In resting CEF cultures, TPA and other active phorbol diesters have been shown to stimulate 2-deoxyglucose transport within 30 min of addition to the culture medium (Driedger and Blumberg, 1977). TPA was maximally effective at 2×10^{-8} M, and a good correlation was demonstrated between the activity of the phorbol diesters in this system and their *in vivo* tumour-promoting activity. For the non-phorbol promoters inconsistent effects have been noted (Driedger and Blumberg, 1978). In contrast, TPA (1.6×10^{-7} M) inhibits 2-deoxyglucose uptake in human lymphocytes in culture (De Chatelet et al., 1976). This may indicate that the effects of TPA on membrane transport are not simply a reflection of altered membrane permeability.

Ohuchi and Levine (1978a) have shown that TPA stimulates synthesis of prostaglandins (PGs) in the canine kidney cell line, MDCK, by causing deacylation of membrane phospholipids. The release of free arachidonic acid, the precursor of PGs, into the culture medium was also stimulated by TPA. However, the release of free linoleic acid from membranes into culture medium was not affected (Ohuchi and Levine, 1978b). TPA may, therefore, specifically increase the activity of a phospholipase A_2 which has a greater affinity for arachidonate than for linoleic acid ester bonds (Ohuchi and Levine, 1978b). The

release of arachidonic acid induced by TPA can be inhibited by indomethacin (Ohuchi and Levine, 1978b), and this may explain the weak anti-promoting activity of this compound. The stimulation of PG synthesis may be a general response of cells to promoter treatment. Similar effects are induced by TPA in cultured mouse epidermal cells, CEFs and other cell types (Weinstein et al., 1979; Furstenberger et al., 1982b). Non-promoting diterpene esters are unable to affect the rate of PG synthesis (Weinstein et al., 1979).

It is generally accepted that TPA does not form covalent adducts with cellular DNA (Boutwell, 1974; Poirier et al., 1975) and it is non-mutagenic (Lankas et al., 1977; Soper and Evans, 1977). However, there have been conflicting reports on the capacity of TPA to induce sister chromatid exchanges (SCE) in various cell types. The role of SCE in carcinogenesis has not been clearly identified, although they certainly reflect a type of damage or rearrangement of the genetic material (Nagasawa et al., 1983). Cells from patients with various hereditary 'cancer-prone' syndromes are known to have elevated levels of this type of chromosome damage (Chaganti et al., 1974; Raj and Heddle, 1980; Shiloh et al., 1985).

TPA has been reported to increase the frequency of spontaneous SCE in Chinese hamster V79 cells (Kinsella and Radman, 1978), and in mouse C3H/10T½, mouse 3T3 and Chinese hamster ovary (CHO) cells (Nagasawa and Little, 1981). That the elevation of SCE may be involved in tumour promotion was supported by the finding that 4-OMeTPA, the weakly promoting analogue of TPA had no effect on SCE levels in V79 cells (Kinsella and Radman, 1978). Conversely, other investigators have been unable to demonstrate this effect for TPA in either V79 or CHO cells (Loveday and Latt, 1979; Thompson et al., 1980; Dewdney and Soper, 1984).

In V79 cells, unusually, SCE induction by TPA was not accompanied by an increase in structural chromosomal damage (chromosome aberrations, CA) (Kinsella and Radman, 1978). However, in cultured human lymphocytes TPA (1.6×10^{-8} M and 1.6×10^{-7} M) has been shown to induce both CA and SCE (Emerit and Cerutti, 1981). Most chemicals and physical agents capable of causing CAs in mammalian cells are also carcinogens (Auerbach, 1976; Carrano et al., 1978). Furthermore, CAs are found in many types of cancerous growth and in an increasing number of cases there is a specific chromosomal abnormality associated with a particular form of neoplastic growth (Rowley, 1984). Thus, the observation of the induction of chromosomal damage by TPA could have implications with respect to the possible mechanism(s) of tumour promotion.

A model to explain how promoters might produce chromosomal damage has been proposed by Emerit and Cerutti (1981, 1982), and depends on the production of activated forms of oxygen. Support for this hypothesis has come from other observations. TPA has been shown to stimulate radical production in human polymorphonuclear leucocytes (PMN), by evoking a typical phagocytic burst of activity (Goldstein et al., 1979; Witz et al., 1980; Kensler and Trush, 1981). This effect is rapid, with the peak response occurring within 5 min of TPA (1.6×10^{-7} M) addition (Kensler and Trush, 1981). The ability of a series of phorbol diesters to stimulate free radical production by PMNs correlates with their tumour promoting activity in mouse skin (Goldstein et al., 1981). The non-phorbol tumour promoters teleocidin and lyngbyatoxin A are also capable of eliciting this rapid response (Formisano et al., 1983). Since leucocytes are part of the inflammatory state induced by TPA in mouse skin (1.3), their ability to generate free radicals may contribute to promotion. However, as

recently TPA has been shown to stimulate radical production in cultured mouse epidermal cells(Fischer and Adams, 1985), it may be not be necessary to include leucocytes in a free radical mechanism of tumour promotion.

Concurrent with the stimulation of free radical production and the induction of chromosome damage, TPA has been shown to reduce the levels of superoxide dismutase (SOD) in both cultured mouse epidermal cells (Colburn et al., 1979) and human leucocytes (Kinsella et al., 1983). As SOD is part of the cellular defence against free radicals, these findings increase the possibility that promoters may induce damage through an indirect mechanism (Emerit and Cerutti, 1981).

It is difficult to envisage a reversible mechanism of promotion including these permanent changes in chromosomes. However, it has been suggested that the initial effects of the increase in free radicals and the lowering of protective enzyme levels are on cell proliferation (Oberley et al., 1981) and on cell differentiation (Oberley et al., 1980). It is more likely that this combination of effects is more important to the late or final stages of promotion (Cerutti, 1985), a view supported by inhibitor studies. Effective inhibitors of Stage II promotion (1.4) suppress both TPA-induced chromosomal damage and free radical production (Witz et al., 1980; Kensler and Trush, 1981; Slaga et al., 1982). Additionally, Cu DIPS, a low molecular weight copper complex with SOD-mimetic activity, has been shown to inhibit both TPA-induced ODC activation and tumour promotion in mouse skin (Kensler et al., 1983).

1.6 Phorbol Diester Receptor Sites

The phorbol diester tumour promoters induce a large number of biological and biochemical changes both *in vivo* (1.3) and *in vitro* (1.5). In contrast, one target, the cell surface membrane, is proposed as the site of the interaction for these compounds with cells. Circumstantial evidence also suggests that these compounds may act by binding to specific receptor sites. Only extremely low doses are required for the induction of their responses, 10^{-8} - 10^{-6} M *in vitro* and 1.7 x 10^{-8} M per application *in vivo*. While small structural modifications in the TPA molecule can markedly affect its activity (Hecker, 1978).

Initial attempts to demonstrate specific binding for the phorbol diesters using radioactive ligands, such as ^{3}HTPA and ^{3}HPDD, were unsuccessful (Helmes et al., 1974; Kubinski et al., 1974), probably due to the high lipophilicity of these particular diesters (Jacobson et al., 1975). The introduction of a less lipophilic derivative, ^{3}H phorbol 12,13 dibutyrate (^{3}HPDBu) has now permitted the demonstration of specific phorbol diester binding to cultured cells (Driedger and Blumberg, 1980) and to other tissues (Delclos et al., 1980; Dunphy et al., 1980; Shoyab and Todaro, 1980). Consistent with their effects in cell culture systems, specific phorbol diester binding sites have been found in virtually all tissues with the exception of erythrocytes (Shoyab et al., 1981).

In contrast to the behaviour of carcinogens, the binding of the phorbol diesters is reversible (Driedger and Blumberg, 1980). Depending on the system, the dissociation constant for their binding ranges from 7-50 nM, and the number of binding sites ranges from 1.5-2.0 pmol/mg protein or 0.5-10 x 10^{5} sites/cell (Blumberg et al., 1983). As in all the systems examined, TPA has a higher binding affinity than ^{3}HPDBu, the receptor being measured should, perhaps be regarded as a TPA receptor (Blumberg et al., 1983).

The pharmacological evidence that the binding detected by ^{3}HPDBu mediates both tumour promotion in mouse skin and the biological response *in vitro* is strong. Excellent quantitative agreements have been demonstrated between the potency of the phorbol diesters to induce specific effects and their ability to inhibit ^{3}HPDBu binding in a variety of cell systems (Blumberg et al., 1984). Additionally, teleocidin and lyngbyatoxin, potent mouse skin tumour promoters structurally dissimilar to the phorbol diesters, also inhibit ^{3}HPDBu binding with nanomolar affinity (Umezawa et al., 1981; Collins and Rozengurt, 1982). However, as anthralin and other phenols with weak promoting activity fail to inhibit ^{3}HPDBu binding (Blumberg et al., 1983) this may not provide a general assay for tumour-promoting agents. Mezerein, the potent Stage II promoter, shows a markedly lower affinity for the receptor than does TPA in most systems (Driedger and Blumberg, 1980). This implies that additional targets may exist which elicit the responses important to the Stage II of promotion.

^{3}HPDBu binding is not significantly affected by hydroxyurea, cycloheximide or sodium fluoride (Shoyab and Todaro, 1980). It does not, therefore, require DNA, RNA or protein synthesis or metabolic energy. Inhibitors of mouse skin tumour promotion, including retinoic acid and dexamethasone, also have no effect (Shoyab and Todaro, 1980). They must induce their inhibitory actions through mechanisms other than the inhibition of phorbol diester binding.

Various workers have attempted to identify the endogenous compound(s) usually interacting with these receptors. Structural analogies have been proposed between the phorbol diesters and cortisol (Wilson and Huffman, 1976), prostaglandins (Symthies et al., 1975) and polyunsaturated fatty acid methyl esters (Rohrschneider and Boutwell, 1973b). In none of these cases has an analogue of comparable potency

and with the ability to mimic the range of action of the phorbol diesters been identified. As the effects of phorbol diesters on cells closely resembles those of a number of naturally occurring polypeptides such as thrombin, epidermal growth factor (EGF) and platelet-derived growth factor, these have also been proposed as the endogenous activators usurped by the phorbol diesters (Mufson, 1984). Again, all of the actions of TPA are not shared by any of these factors (Lee and Weinstein, 1978), and there are cell types which appear to lack EGF receptors that still respond to TPA (Pruss and Herschman, 1977; Greenbaume et al., 1983). The binding studies of Dunphy et al. (1980) suggest that the phorbol diester receptor is not analogous to a hormone receptor. These authors showed that the levels of ^{3}HPDBu binding in the mouse brain (30 pmol/mg protein) were markedly higher than those for the neurotransmitters ($\sim$ 2 pmol/mg protein) or for other hormones. Furthermore, in mouse fibroblasts EGF does not compete with ^{3}HPDBu for its binding sites (Shoyab and Todaro, 1980).

Based on the similarity in the phenotypes induced by Rous sarcoma virus (RSV) and TPA in CEF (Driedger and Blumberg, 1977) it has been proposed that the immediate response of the phorbol diester receptor interaction could be a change in kinase or phosphatase activity or specificity (Blumberg, 1981). The mechanism of transformation by RSV has been identified to be by the introduction into the cell of a tyrosine kinase, apparently analogous to a normal cellular enzyme but expressed at an enhanced level (Erikson et al., 1981). Indeed the properties of the phorbol diester receptor shows marked similarities to those of protein kinase C (PKC) described by Takai et al. (1979a, 1979b). This is a Ca^{2+}- and phospholipid-dependent protein kinase for which diacylglycerol,

transiently produced during phosphatidylinositol breakdown in a signal-dependent manner, is the endogenous activator (Takai et al., 1979a, 1979b).

There are a growing number of reports to suggest that some, if not all, of the pleiotropic actions of the tumour promoting phorbol diesters may be mediated through PKC. TPA and other phorbol diesters have been shown to directly activate PKC both *in vivo* and *in vitro* by substituting for diacylglycerol, without eliciting the breakdown of phosphatidylinositol (Castagna et al., 1982; Yaminishi et al., 1983). The ability of a series of phorbol diesters to activate PKC correlates with their tumour promoting activity (Castagna et al., 1982). Other promoters, such as teleocidin and lyngbyatoxin, previously shown to inhibit ^{3}HPDBu binding also activate PKC, while anthralin and other weak promoters have no effect (Kikkawa et al., 1983). *In vitro*, the binding of ^{3}HPDBu to PKC has an absolute requirement for both Ca^{2+} and phospholipid. This has led to the proposal that the specific phorbol diester receptor is a quarternary complex involving Ca^{2+}, phospholipid, PKC and an active phorbol diester (Kikkawa et al., 1983). Of a series of phospholipids tested, phosphatidylserine was shown to be the most effective (Kikkawa et al., 1983). This is identical to the phospholipid requirement of PKC activated *in vivo* by diacylglycerol (Kaibuchi et al., 1981). *In vivo*, Ashendel et al. (1983) have shown that the binding of ^{3}HPDBu to its receptors in various mouse tissues also required Ca^{2+} and phospholipid.

There are other similarities between the ^{3}HPDBu receptor and PKC which suggest that they may be the same. The tissue distribution of the ^{3}HPDBu binding protein has been found to parallel that of PKC (Nishizuka et al., 1979; Shoyab et al., 1981). Both have been found in inactive forms in the cytosol of mammalian cells, and are activated by

reversible association with membrane phospholipids in the presence of Ca^{2+} (Takai et al., 1979b; Kraft and Anderson, 1983). Additionally, in subcellular fractionation studies, the ^{3}HPDBu binding protein from rat brain has been shown to copurify with PKC (Neidel et al., 1983). In kinetic studies the apparent dissociation constant of ^{3}HPDBu binding, 8 nM, is identical to its apparent activation constant for PKC in homogenised rat brain preparations (Kikkawa et al., 1983). One difference, however, is apparent. Phorbol diester activation of PKC *in vivo* is sustained and not subject to feedback control by the cyclic nucleotides (Yaminishi et al., 1983). This is probably because the phorbol diesters intercalate into the cell membrane (Castagna et al., 1982; Delclos et al., 1983), and are metabolized at a much slower rate than diacylglycerol (Nishizuka, 1983).

The physiological role of PKC is as yet unknown, although it may be involved in cellular proliferation (Nishizuka, 1984). It has a broad substrate specificity, and has been found to phosphorylate seryl and threonyl residues but not tyrosyl residues in many endogenous proteins. Perhaps of most importance are the recent reports that PKC activation causes phosphorylation of the EGF receptor (Cochet et al., 1984) and the β-subunit of the insulin receptor (Takayama et al., 1984), preventing the binding of the endogenous activators. Similarly, TPA has been reported to induce phosphorylation of EGF receptors in cultured human epidermoid cells (Iwashita and Fox, 1984) and insulin receptors in cultured human B-lymphocytes (Jacobs et al., 1983).

Consistent with the diacylglycerols being the endogenous analogues of the phorbol diesters, they have been shown to competitively inhibit ^{3}HPDBu binding to its receptor site (Sharkey et al., 1984). TPA has a diacylglycerol-like structure (Nishizuka, 1984) and *in vitro* the synthetic diacylglycerol derivative, 1-oleoyl-2-

acetylglycerol (OAG) has been shown to induce similar, rapid biochemical reponses to the phorbol diesters in several systems. OAG induces ODC activity in cultured rodent cells (Jetten et al., 1985), and terminal differentiation of HL-60 cells (Ebeling et al., 1985; Kreutter al., 1985). PKC activation may also be involved in the production of free radicals by PMNs treated with phorbol diesters. OAG has been shown to both induce phosphorylation of marker proteins and stimulate superoxide anion production in human neutrophils (Robinson et al., 1984; Fujita et al., 1985). Superoxide radicals generated in a xanthine-xanthine oxidase system did not induce phosphorylation of these marker proteins (Fujita et al., 1985). A number of phorbol diester-induced changes can thus be explained by activation of PKC. However, the critical protein changes in promotion are yet to be identified.

1.7 Mutations and Mutagens

1.7.1 Gene Mutations and Their Detection

The material of inheritance of almost all living organisms is deoxyribonucleic acid (DNA). The normal DNA molecule is a double helix, consisting of a pair of polynucleotide strands coiled around each other. These are held together by hydrogen bonds between pairs of complementary purine (adenine (A) and guanine (G)) and pyrimidine (cytosine (C) and thymine (T)) bases. The only base pairs usually permitted by hydrogen bonding and steric hindrance are A-T and G-C. In all cells, prior to division, DNA is replicated in a semi-conservative manner. The two strands separate and each serves as a template for the formation of a new daughter strand by normal AT and GC base pairing.

The order of bases on one strand of the cells DNA constitutes a genetic message which contains all the information necessary to determine the specific structure and functions of that cell. This genetic information is translated into polypeptide chains (proteins) in a complex process involving messenger RNA, ribosomes and transfer RNA (Watson, 1976). Each amino acid component of a particular polypeptide is specified by a triplet of successive bases (a codon). A segment of DNA in which the order of bases ultimately determines the sequence of amino acids in a single polypeptide chain is called a gene.

Gene mutations are heritable alterations to DNA affecting a single gene. This may occur spontaneously or be caused by external agents - mutagens. In molecular terms gene mutations consist of the substitution, addition or deletion of one or more base-pairs in DNA. Mutations due to substitutions are known as base-pair substitutions and those due to additions or deletions as frameshifts.

A base-pair substitution mutation occurs when a wrong base is inserted which then pairs with its natural partner during DNA replication. In this way, a new pair of incorrect bases is inserted into DNA. On translation, this results in the alteration of only one amino acid within a particular protein. In contrast, when a frameshift mutation occurs the subsequent genetic message becomes disrupted, leading to the formation of an altered polypeptide. As they cause alterations in the structure of proteins, gene mutations can bring about profound changes in the anatomical and metabolical functions of cells. Certain gene mutations affecting dispensible, enzymatic functions can be detected if the resultant mutant organism possesses a phenotypic trait which allows it to be distinguished from normal (wild-type) members of a population.

Mutations of specific sites arise in either a 'reverse' (mutant to wild-type) or a 'forward' (wild-type to mutant) direction. Reverse mutation is generally studied in mutant cells containing known base-pair substitution or frameshift mutations. In these cells, normal functional activity can be restored following a new substitution, or a second addition or deletion close to the first. Thus, a reverse mutation requires a highly specific type of DNA interaction. The Ames test (Ames et al., 1973), which is used to screen environmental chemicals for mutagenicity, is the best known assay system measuring reverse mutations. It is based on the reversion of histidine-requiring *Salmonella typhimurium* bacteria to prototrophy (growth factor independence).

Forward mutations may arise from substitutions, additions or deletions of the bases of a gene, or from the deletion of an entire gene and/or neighbouring genes. These can be detected when there is a change in an enzymatic function resulting in auxotrophy (growth factor

dependence), or in resistance to various chemical or physical agents. Numerous genetic markers, based on forward mutations to drug resistance, are employed in mammalian cell mutation assays (Puck and Ham, 1968; Gupta and Singh, 1982; Adair and Carver, 1983). However, the most commonly used markers are resistance to the antimetabolite 6-thioguanine (Arlett, 1977b; Bradley et al., 1981; Hsie et al., 1981), to the steroid compound ouabain (Baker et al., 1974; Cole and Arlett, 1976; Gupta and Siminovitch, 1980), or to high concentrations of the nucleoside thymidine (Anderson and Fox, 1974; Clive, 1977).

Whatever the cell type employed, mutation assays always comprise the same three basic steps - cell treatment, mutation expression and mutant selection. During the cell treatment a standardised cell population is exposed to a mutagen under a well defined and reproducible set of conditions. The expression time is the period between the initiation of cell treatment and the addition of the selecting agent. This period is necessary to allow induced DNA lesions to yield mutations and for the transcription of mutated genes. It also provides time for the disappearance of existing gene products from mutant cells, by dilutions at successive cell divisions and by normal degradation. Specific mutants are selected in the final step, by imposing growth conditions on the cell populations which kill or inhibit normal cells but which permit mutants to give rise to macroscopic colonies.

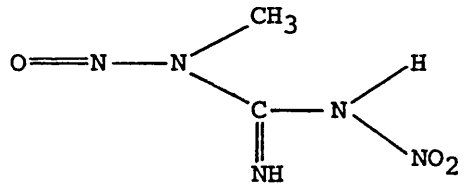
The densities of the cells on the plates during mutant selection is an important factor in mammalian cell mutation assays. Many mammalian cell mutants engage in metabolic cooperation when they are in contact with normal cells. This is a process whereby mutant cells can receive certain substances from normal cells. In the case of enzyme deficient mutants these substances are presumed to be enzyme

products which render the cells phenotypically normal and, therefore, unselectable as mutants (Simons, 1974; Abbondandolo, 1977). In mutation assays this leads to a reduced yield of mutant colonies and a consequence low value for the mutation frequency. This problem can be overcome by selecting for mutants at cell densities at which cell-cell contacts are not permitted.

The biochemical bases of the drug resistance markers to be employed during these investigations are detailed later.

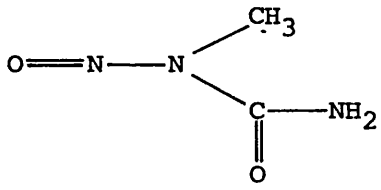
1.7.2 Simple Alkylating Agents

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)



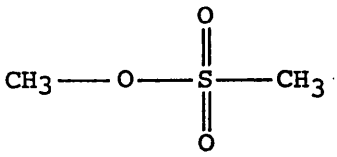
Mol. Wt. 147.1

N-methyl-N-nitrosourea (MNU)



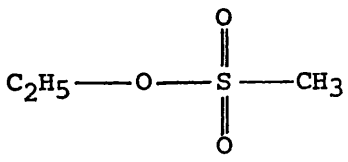
Mol. wt. 103.1

Methylmethane sulphonate (MMS)



Mol. Wt. 110.1

Ethylmethanesulphonate (EMS)



Mol.wt. 124.2

The alkylating agents represent the largest class of mutagens present in man's environment (Fishbein et al., 1970). The four agents used in these investigations are all monofunctional alkylators, i.e. they only

donate a single alkyl group to a nucleophilic site within a biological macromolecule. The positions of the normal DNA bases susceptible to alkylation are the N1, N^2, N3, O^6, N7 and C8 of guanine; the N1, N3, N^6, N7 and C8 of adenine; the N3 and N^4 of cytosine and the N3 and O^4 of thymine (Drake and Baltz, 1976). The superscript notation signifies exocyclic sites and the nonsuperscript notation endocyclic sites. The spectrum of DNA alkylation produced by these agents varies with their chemical nature. Reagents of low nucleophilicity (classed as SN1 agents), typified by MNNG and MNU, react relatively more extensively at O-atom sites than do those of high nucleophilicity (SN2 agents), such as MMS (Lawley, 1974). EMS, which is classified as a borderline SN1/SN2 agent (Sega, 1984) fills an intermediate position in this respect. The agents used in these investigations thus represent a broad range of reactivities.

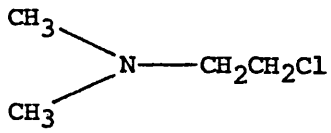
Alkylation of DNA can produce both potentially miscoding (mutagenic) lesions and potentially lethal lesions. O^6 alkylG and O^4 alkylT probably represent the most important DNA lesions with respect to the induction of gene mutations, and cancer, by simple alkylating agents (Lawley, 1974; Singer, 1975; Pegg, 1977). These alkylated bases almost certainly undergo mispairing with thymine and guanine, respectively, to generate GC to AT and TA to GC substitutions (Drake and Baltz, 1976). Alkylation at the N7 position of guanine generally correlates with the lethality of these agents (Lawley, 1974; Auerbach, 1976). The internucleotide phosphates of DNA are also potential sites of alkylation (Lawley, 1974; Singer, 1975). However, the significance of this type of DNA lesion to either the mutagenic or the lethal effects of these particular alkylating agents remains uncertain (Gichner and Veleminsky, 1982; Sega, 1984).

Alkylating agents may also induce mutation indirectly by a

misrepair mechanism (Drake and Baltz, 1976). This occurs when a premutational lesion interrupts daughter strand elongation during DNA replication. The gap produced in the daughter strand becomes a substrate for an enzymatic error-prone post-replication DNA mechanism (Heidelberger, 1975; Hart et al., 1978). When these gaps are filled either base-pair substitutions or frameshift mutations can be generated. The ratio of directly to indirectly induced mutations depends upon both the alkylating agent and the particular organism. For example, in *Escherichia coli* only about 30% of EMS mutagenesis occurs indirectly, while in yeasts EMS mutagenesis occurs almost exclusively by this mechanism (Drake and Baltz, 1976).

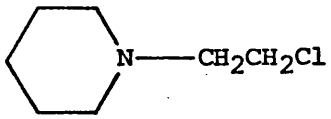
1.7.3 Dialkylaminoalkyl Chlorides

Dimethylaminoethyl (DMAE)



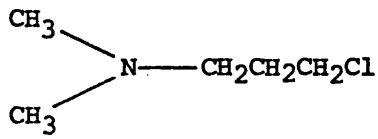
Mol. Wt. 107.6

1(2-Chloroethyl)piperidine (CEP)



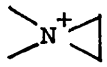
Mol. Wt. 147.6

Dimethylaminopropyl (DMAP)



Mol. Wt. 121.6

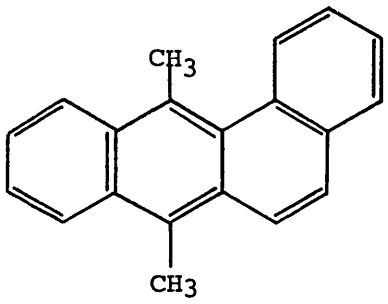
Aziridinium ion



These are monofunctional alkylating agents extensively employed in the pharmaceutical industry for the addition of side chains. At neutral pH the dialkylaminoethyl-like members of the series probably exist in equilibrium with a cyclic aziridinium ion form. The aziridinium ion is a potent alkylator of functional nucleophilic groups (Auerbach, 1976).

No chronic toxicity studies have been performed on these compounds. However, they have been detected as base-pair substitution mutagens in bacteria (Soper et al., 1979) and mammalian cells (Thompson et al., 1981). In both studies, the mutagenic activity of a series of these compounds was correlated with their ability to form the aziridinium ion. Two of the compounds used in these investigations, DMAE and CEP, can form this ionic species, while DMAP cannot.

1.7.4 7,12-Dimethylbenz [a] anthracene (DMBA)



Mol Wt. 256.8

DMBA is a known carcinogen frequently used as an initiating agent in the mouse skin model of two-stage tumour induction. In common with other polycyclic hydrocarbons, it requires metabolic activation to produce the active species which interacts with DNA (Miller and Miller, 1976). Different activating systems may produce different mutagenic species (Arcos and Argus, 1974; Sims, 1980; Singer and Kusmierek, 1980). However, these all appear to induce mutations by a similar mechanism. Following its reaction with DNA it is proposed that the bound mutagenic species may intercalate between the base pairs causing distortion and localised unwinding of the helical DNA structure (Arcos and Argus, 1974). Mutations then arise by an indirect mechanism, as the distorted DNA becomes the substrate for error-prone repair systems.

CHAPTER 2. MATERIALS AND METHODS

2.1 Equipment

Laminar flow hood. During the course of this work two different laminar flow hoods were used for all aseptic preparations and cell culture work.

(i) 2 m, horizontal displacement type (Fell Clean Air (1971) Ltd., Newhaven, Sussex).

(ii) 1.3 m, vertical displacement type, Class I biological safety cabinet, Microflow model No. 20229 (MDH Ltd., Andover, Hampshire).

All the surfaces of the hoods were swabbed with 70% alcohol before and after use.

Incubator. LEEC PF2 anhydric incubator with forced air circulation (Laboratory and Electrical Engineering Company, Nottingham). The thermostatic controls were adjusted to maintain a temperature of 37.5°C.

Bench centrifuge. MSE Minor, model No. S-61 (MSE Scientific Instruments, Crawley, Sussex).

Pipette fillers. For use with graduated glass pipettes, Pi-pump suction pumps (Glasfrin Giessen via Jencons Ltd.) were used for all pipette filling. Pipetting by mouth was never undertaken.

Adjustable replicating pipettes.

Pipetman P20	2-20 µl
Pipetman P200	20-200 µl
Pipetman P1000	200-1000 µl
Pipetman P5000	1000-5000 µl

Pipetman pipettes with tips were obtained from Anachem Ltd., Luton, Bedfordshire. All pipettes were checked at regular intervals to ensure

they were delivering accurately and reproducibly. A gravimetric method was used in which 10 replicates of a set volume of water at 20°C were each weighed on an analytical balance.

Liquid nitrogen freezers. Stock cultures of cells were stored in 2 ml ampoules in one of two types.

i) Union Carbide model no. LR-33-10 (Union Carbide UK Ltd., Cleveland), in which the ampoules are held on canes in the liquid N_2 refrigerant or its overlying vapour.

ii) Union Carbide model no. LR40, in which the ampoules are shelved in the vapour phase of the liquid N_2.

Freezing unit. Union Carbide BF-6 biological freezer, a plug-type device, designed for use with the LR-33-10 freezer. This unit is capable of cooling up to eight 2 ml ampoules at between 0.5°C and 7°C min^{-1} to below -70°C.

Haemocytometer. Standard double grid improved Neubauer-type blood cell haemocytometer, with coverslips (Fisons Ltd., Loughborough).

Microscopes

(i) For the examination of growing cell cultures under phase contrast and for haemocytometer counting an inverted biological microscope Wild M40 (Wild Heerburgg Ltd., Heerburgg, Switzerland) was employed. This instrument fitted with appropriate condensers and X10 and X20 objectives gives magnifications of X187 and X375, respectively.

(ii) Binocular dissecting microscope. Standard microscope model (C. Baker Ltd., London), which allows subject examination at X20.

Disposable cell culture plasticware. 25 cm^2 tissue culture (T/C) flasks, 100 mm x 16 mm T/C tubes with screw caps, 50 mm x 13 mm and 90 mm x 16 mm T/C petri dishes with triple vented lids, were all of tissue culture grade polystyrene and obtained ready sterilised, from

Sterilin Ltd., Feltham. Ampoules for the storage of cells in liquid N_2, 2 ml volume, polypropylene, with silicone rubber-lined screw tops, were obtained, ready sterilised from Sterilin.

30 ml polystyrene universal containers with screw caps were obtained ready sterilised from Sterilin.

Glassware. 125 ml and 500 ml bottles for the storage of solutions and media were obtained with caps, from Flow Laboratories Ltd., Irvine, Scotland.

The 150 ml soda glass 'medical' flat bottles in which cells were routinely grown, all other bottles and caps, and general laboratory glassware were obtained from Fisons Ltd.

2.2 General Methods

2.2.1 Sterilisation by membrane filtration. All heat labile liquids, solutions and media were sterilised by membrane filtration.

(i) Small volumes were filtered through autoclave sterilised 25 mm Swinnex units (Millipore UK Ltd., London), fitted with 0.2 µm or 0.45 µm pore size Sartorius membrane filter discs (V.A. Howe and Co. Ltd., London).

(ii) Small volumes of hazardous solutions were filtered from luer-locked syringes through disposable Millex-GS units (Millipore), fitted with membrane filters of 0.2 µm pore size.

(iii) Non-aqueous solvents, incompatible with cellulose nitrate filters, were filtered through disposable Millex-FG units (Millipore), fitted with Fluorophore hydrophobic membrane filters of 0.2 µm pore size.

(iv) Large volumes of solutions and media, greater than 250 ml, were filtered from a positive pressure apparatus (AB Stalmanufaktur, Stockholm, Sweden) through an autoclave sterilised 47 mm Swinnex unit fitted with an AP25 prefilter (Millipore), a Dacron filter separator (Millipore) and a 0.2 µm pore size membrane filter disc (Sartorius). Filtration under positive pressure was used in preference to filtration under negative pressure as it prevents frothing and minimises the loss of CO_2 from bicarbonate containing media.

2.2.2 Recycling of glassware. Immediately after use all glassware was rinsed with tap water, and, except for pipettes, processed as follows.

(i) All articles were soaked in a hand-hot 2% v/v solution of RBS25 detergent (Fisons Ltd.) for 30 min.

(ii) Thoroughly cleaned, using a China brush on all surfaces under running water.

(iii) Rinsed in three changes of tap water, being left for 30 min in the last rinse.

(iv) Rinsed in three changes of distilled water, again leaving for 30 min in the last rinse.

(v) Finally, all articles were rinsed and left for at least 30 min in a large volume of freshly collected glass distilled water.

After drying in a hot air oven, all items were foil capped and sterilised at 160°C for a minimum of one hour. Graduated glass pipettes were rinsed in a pipette washer with tap water, for at least 30 min, and then rinsed as above, from (iii). After drying in a hot air oven, pipettes were put into metal canisters and sterilised at 160°C for a minimum of one hour.

2.2.3 Recycling of non-glass items. Specifically the tips for the replicating pipettes, bottle caps and membrane filter holders. Immediately after use these were rinsed with tap water and cleaned by boiling in three changes of single distilled water. After this they were rinsed as for glassware (from 2.2.2(iii)). After drying in a warm air oven, all items were sealed in autoclave bags (DRG Hospital Supplies, Bristol) and sterilised by autoclaving at 121°C for 15 mins.

2.2.4 Gases and gassing procedures.

(i) Cylinders of N_2, CO_2 and 5% CO_2 in air mixture (5% CO_2 and 20% O_2 in N_2) were all obtained from British Oxygen Company, Bristol. CO_2 and 5% CO_2 in air mixture were piped to each work station from a central holding reservoir.

(ii) Gas flowmeter, floating-needle type, 0.1-1.0 $lmin^{-1}$ (Rotameter Manufacturing Co. Ltd., Croydon), calibrated for use with CO_2.

(iii) Incubation boxes, rigid clear plastic boxes, 3.25 litre volume (A. Gallenkamp and Co. Ltd., London).

(iv) Gas-tight tape, British standard vinyl tape, 2.5 cm wide (Intech Tapes Ltd., Manchester).

All the cell culture media used contain a bicarbonate pH buffer system designed to equilibrate with 5% CO_2 in air. To achieve this all gas-tight culture bottles and flasks were charged with such a mixture, introduced at a low flow rate through a sterilised Pasteur pipette plugged with non-absorbent cotton wool. Cell culture dishes were placed in incubation boxes which were flushed with 150 ml of CO_2 from a metered supply, through a plugged Pasteur pipette. The boxes were then sealed with gas-tight tape. When only a few plates were put into a particular box an open 50 ml beaker of sterile water was included to prevent excessive evaporation of the culture medium.

2.2.5 Siliconising of Pasteur pipettes. Pasteur pipettes were coated by twice drawing up dimethylsilane solution (BDH Chemicals, Poole, Dorset) into them. They were allowed to air dry, and then immersed in a large volume of freshly collected distilled water for a minimum of 30 min. The siliconised pipettes were dried in a warm air oven, sealed in autoclave bags and sterilised by autoclaving at 121°C for 15 min.

2.3 Cell Culture Materials

2.3.1 Water. Double glass distilled water (DDH_2O) was used in the preparation of all solutions and media. This was obtained from a 4 litre hr^{-1} bi-distillation Fistream still, model 2903 (Fisons Ltd.) fitted with a Fistream pre-deionizer (Fisons Ltd.) which was changed at regular intervals. DDH_2O was sterilised by autoclaving at 121°C in 100 ml, 500 ml and 1 litre quantities in glass bottles, for appropriate times. Sterile DDH_2O produced in this way has a pH of 4.5.

2.3.2 Balanced salt solution. Dulbecco's phosphate buffered saline was routinely used, either with or without added calcium and magnesium ions, solutions designated PBS and PBS(A), respectively.

Composition: (Dulbecco and Vogt, 1954).

Component	gl^{-1} DDH_2O	
NaCl	8	
KCl	0.2	PBS(A)
Na_2HPO_4	1.15	pH 7.3
KH_2PO_4	0.2	
$CaCl_2.2H_2O$	0.132	
$MgCl_2.6H_2O$	0.1	

PBS
pH 7.4

Sterile PBS(A) was prepared by dissolving one Dulbecco A tablet (Oxoid Ltd., London) in 100 ml of freshly collected DDH_2O and autoclaving for 15 min at 121°C. PBS was prepared by the aseptic addition of 0.5 ml Dulbecco B solution (Oxoid Ltd.) to 100 ml of sterile PBS(A). Both PBS and PBS(A) were stored at room temperature, for a maximum of one month.

2.3.3 Additives for Cell Culture Media

(i) Antibiotic solution, penicillin (5,000 IU ml^{-1}) and streptomycin (5,000 µg ml^{-1}) sterile solution was obtained from Flow Labs Ltd. in 100 ml unit quantities. This was subdivided into 15 ml volumes and stored frozen in plastic universals at -20°C, for a maximum of one year.

(ii) L-Glutamine solution was obtained as a sterile 200 mM solution in 100 ml unit quantities from Flow Labs. Ltd. This was subdivided into 15 ml volumes and stored frozen in plastic universals at -20°C, for a maximum of one year.

(iii) L-Proline solution was prepared at a concentration of 100 mM from chromatographically homogeneous L-proline (cell culture tested, Sigma Chemicals Ltd., Poole, Dorset) in DDH_2O, and sterilised by membrane filtration. 5 ml volumes of this solution were stored in glass bijou bottles at -20°C, for a maximum of one year.

(iv) L-Cysteine solution was prepared as a 40 mM solution of L-cysteine hydrochloride (cell culture tested, Sigma) in DDH_2O and sterilised by membrane filtration. 10 ml volumes of this solution were stored in plastic universals at -20°C, for a maximum of one year.

(v) Glutathione (GSH) solution was prepared as a 20 mM solution of GSH, reduced form (cell culture tested, Sigma) in DDH_2O and sterilised by membrane filtration. 10 ml volumes of this solution were stored in plastic universals at -20°C for a maximum of one year.

(vi) Sodium bicarbonate was obtained as a sterile 7.5% w/v $NaHCO_3$ solution in 100 ml unit quantities from Flow Labs Ltd. This

solution was stored at room temperature for a maximum of one year.

(vii) Thymidine solution was prepared as a 3 mM solution of chromatographically homogeneous thymidine (BDH Chemicals) in DDH_2O and sterilised by membrane filtration. 5 ml volumes were stored in glass bijou bottles at -20°C for a maximum of one year.

2.3.4 Media

(i) Ham's F10 medium, Eagle's minimal essential medium with Earle's salts, and 199 medium with Earle's salts were all obtained as sterile 10 x liquid concentrates with phenol red but without L-glutamine and $NaHCO_3$ from Flow Labs Ltd. in 100 ml units. These were stored at 4°C for a maximum of one year.

500 ml volumes of single strength media were aseptically prepared from these concentrates, with other media components added in the order given (Table 2.1). Once prepared the single strength media were stored in 100 ml volumes at 4°C and used within 4 weeks.

(ii) Ham's F10 medium without hypoxanthine, thymidine and $NaHCO_3$ was obtained in powder form from Flow Labs Ltd. and stored at 4°C for a maximum of one year. Powder to prepare 1 litre of Ham's F10 medium without hypoxanthine (F10(-HX)) was added to approximately 800 ml of freshly collected DDH_2O. The powder was allowed to dissolve and the following components added in the order given.

Table 2.1 Preparation of single strength medium from 10X concentrates.

COMPONENT (ml)	MEDIUM					
	F10 (a)	EMEM (b)	EMEM with L-cysteine (EMEM+LC)	EMEM with GSH	199 (c)	199 with L-cysteine (199+LC)
Sterile DDH$_2$O	428	425	422.5	420	425.5	423
10X medium concentrate	50	50	50	50	50	50
Antibiotic solution	5	5	5	5	5	5
L-proline solution	1	1.5	1.5	1.5	-	-
L-glutamine solution	7.5	5	5	5	5	5
L-cysteine solution	-	-	2.5	-	-	2.5
GSH solution	-	-	-	5	-	-
$NaHCO_3$ solution	8	13.5	13.5	13.5	14.5	14.5

(a) Ham's F10 medium (Ham, 1963).
(b) Eagle's minimal essential medium (Eagle, 1959).
(c) 199 medium (Morgan et al., 1950).

Antibiotic solution	10 ml
L-proline solution	2 ml
L-glutamine solution	10 ml
Thymidine solution	1 ml
$NaHCO_3$ solution	16 ml

The medium was made up to 1000 ml with DDH_2O, rendered slightly acidic by bubbling with CO_2 and sterilised under positive pressure membrane filtration (2.2.1). The sterilised medium was stored in 100 ml volumes at 4°C and used within 4 weeks.

2.3.5 Trypsin solutions.

Trypsin (1:250) was obtained as a sterile 2.5% w/v solution in Hank's balanced salt solution without calcium, magnesium and phenol red, in 100 ml unit quantities from Flow Labs Ltd. This was aseptically diluted to working strengths of 0.05% w/v, 0.025% w/v or 0.025% w/v with 0.02% w/v EDTA with PBS(A). These solutions were stored at -20°C in approximately 16 ml volumes in plastic universals, and used within 4 weeks. Prior to use the solutions were thawed in a 37°C water bath.

2.3.6 Sera

For all the cell lines used the culture medium was supplemented with 5% foetal calf serum (FCS). To achieve this level 5.3 ml of FCS was added to 100 ml of medium immediately before use.

During the course of these studies FCS from four different batches was used. These were selected after the evaluation of FCS samples from a number of different commercial sources for the ability, as a media supplement, to support clonal growth.

Batch 1: Sera Labs Ltd., Crawley	Batch No 001090
Batch 2: Gibco Ltd., Paisley, Scotland	Batch No. U523901D
Batch 3: Sera Labs Ltd.	Batch No. 301110
Batch 4: Gibco Ltd.	Batch No. 40G4136S

Serum from both sources was obtained filter sterilised in 500 ml unit quantities. These were allowed to thaw at 4°C and were then subdivided into 100 ml volumes. These were stored frozen at -20°C, for a maximum of 18 months. As required, 100 ml volumes were withdrawn from stock and allowed to thaw at 4°C. Once thawed the serum was used within 2 weeks.

2.3.7 Cryoprotectants and Solvents

(i) Glycerol, AnalaR grade (Fisons Ltd.) was stored at room temperature, protected from moisture. Prior to use as a cryoprotectant, 2-3 ml volumes were sealed in glass powder ampoules and sterilised by dry heat at 160°C for one hour.

(ii) Dimethylsulphoxide (DMSO), grade 1 (Sigma) was obtained in 100 ml quantities and stored in tightly capped bottles at room temperature. For use as a cryoprotectant DMSO was filter sterilised through 0.2 µm Millex-FG units. As a solvent it was used without sterilisation.

(iii) Acetone, HPLC grade (Fisons Ltd.) was stored at room temperature, protected from moisture. It was used as a solvent without sterilisation.

2.3.8 Stain

For staining colonies attached to plates a 0.5% w/v solution of methylene blue (BDH Chemicals) in 50% methanol was used.

2.4 Mutagens

2.4.1 N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) was obtained in crystalline form (Sigma) and stored in a desiccator at -20°C. Stock solutions of 1 mg ml^{-1} were prepared in DDH_2O. 6 ml aliquots of this were sterilised through 0.2 µm Millex-GS filter units and stored in glass bijou bottles at -20°C, protected from light for a maximum of 4 weeks. Immediately before use stock solutions were thawed in a 37°C water bath and diluted with sterile DDH_2O before addition to cell cultures. The purity of the MNNG used in these studies was assessed by the spectrophotometric method of La Polla et al. (1972) - see Appendix.

2.4.2 N-Methyl-N-nitrosourea (MNU) was obtained in crystalline form, with approximately 25% of its weight made up from a 5% solution of acetic acid as a stabiliser (Sigma). This was stored at 4°C. Solutions were prepared at the appropriate concentrations in culture medium immediately prior to use.

2.4.3 Methylmethane sulfonate (MMS) and ethylmethane sulfonate (EMS) were both obtained as pure liquids (Sigma) and stored at 4°C. Solutions at appropriate concentrations were prepared in culture medium immediately prior to use.

2.4.4 7,12-Dimethylbenz [a] anthracene (DMBA) was obtained in crystalline form (Sigma) and stored at 4°C. Solutions were prepared at appropriate concentrations in DMSO immediately prior to use.

2.4.5 Dialkylaminoalkyl chlorides. Dimethylamino propylchloride (DMAP) was obtained in crystalline form (Aldrich Chemical Co. Ltd).

Dimethylaminoethyl chloride (DMAE) and 1 (2-chloroethyl)piperidine (CEP) were synthesised and chemically characterised in the School of Pharmacy, University of Bath. Solutions of these were prepared, immediately prior to use, at a concentration of 50 mg ml^{-1} in DDH_2O and sterilised through 0.2 μm Millex-GS filter units. Before addition to cell cultures these solutions were diluted in sterile DDH_2O.

2.5 Promoters

2.5.1 12-O-Tetradecanoylphorbol-13-acetate (TPA) was obtained in nitrogen purged glass ampoules and stored at -20°C, protected from light. It has been reported that the activity of this compound may be dependent upon its source (Nagasawa and Little, 1981). The TPA used throughout these studies was therefore obtained from one single supplier from 3 different lots (Sigma).

Batch 1	10 mg	Lot no. 13F-0219
Batch 2	1 mg	Lot no. 52F-0205
Batch 3	15 mg	Lot no. 34F-0682.

Stock solutions were prepared in DMSO at 1 mg ml^{-1}, and subdivided into approximate 0.5 ml aliquots in small glass bijou bottles with foil-lined caps. Each bottle was purged with N_2 before sealing, and stored at -20°C protected from light. Under such storage conditions phorbol esters are reported to be stable for many months (Schmidt and Hecker, 1975).

2.5.2 Benzoyl peroxide (BZP) was obtained from Stieful Research Institute, New York, as a 70% active granular powder moistened with 30% water. To prevent the interference of oxidation products in experimental results, solutions were prepared in acetone at a concentration of 1.5 mg ml^{-1} immediately prior to use.

2.5.3 Formaldehyde (HCHO) was obtained as a 38% w/v solution containing 10-14% methanol as a stabiliser to prevent polymerisation (Fisons Ltd.) and stored at room temperature. Immediately before use this was diluted in sterile DDH_2O before addition to cell cultures.

2.5.4 Linear alkanes. n-Decane, n-dodecane and n-tetradecane were all

obtained as 99%+ pure liquids (Sigma) and stored at room temperature. Immediately before use they were diluted in acetone to a concentration of 0.12M before addition to cell cultures.

2.6 Mutagenesis Assay Selecting Agents

2.6.1 Ouabain (Oua) was obtained as ouabain octahydrate crystals (Sigma) and stored at room temperature. Stock solutions were made at 30 mM or 10 mM in culture medium by dissolving at 50°C, protected from light. Solutions were sterilised by membrane filtration, stored in the incubator at 37.5°C and used within 48 hours. 1 ml of the appropriate stock solution was added to each selection plate with 9 ml of medium to give 3 mM or 1 mM Oua as required.

2.6.2 Thioguanine (TG) was obtained in crystalline form, assay approximately 98% (Sigma) and stored at room temperature. A 10 mM stock solution was prepared in 0.5% w/v aqueous sodium carbonate and sterilised through a 0.2 µm Millex-GS filter unit. 5 ml volumes were stored at -20°C protected from light for a maximum of 4 weeks.

2.6.3 Emetine (Emt) was obtained as emetine dihydrochloride (Sigma) and stored at 4°C. A 10^{-3} M stock solution was made in DDH_2O and sterilised through a 0.2 µm Millex-GS filter unit. 5 ml volumes were stored at -20°C protected from light, for a maximum of 4 weeks. Immediately before use the stock solution was thawed at 37°C and diluted in culture medium before addition to selection plates.

2.6.4 Methylglyoxal bis(guanylhydrazone) (MGBG) was obtained in crystalline form (Sigma) and stored at -20°C. A 10^{-2} M stock solution was made in DDH_2O and sterilised through a 0.2 µm Millex-GS filter unit. 5 ml volumes were stored at -20°C protected from light, for a maximum of 4 weeks. Immediately before use the stock solution was thawed at 37°C and diluted in culture medium before addition to selection plates.

2.6.5 Aminopterin was a gift from Lederle Laboratories and stored at -20°C. A 10^{-4} M stock solution was prepared in a 0.5% w/v aqueous sodium carbonate and sterilised through a 0.2 μm Millex-GS filter unit. 5 ml volumes were stored at -20°C protected from light, for a maximum of 4 weeks.

2.7 Cell Lines

(i) CHO-K1A cells, a subclone of CHO-K1, a L-proline requiring Chinese hamster cell line (Kao and Puck, 1968), were isolated and characterised by Dr. R.S. Dewdney (1982). These cells were used in experiments between passages 30 and 40.

(ii) V79-379A cells, a subclone of V79-1 mature male Chinese hamster lung fibroblasts (Ford and Yerganian, 1958), were obtained from Flow Labs Ltd. These were obtained at an unknown passage number and used in experiments for up to 10 passages after receipt, as recommended by the suppliers.

(iii) Chinese hamster ovary cells, designated CHO(S), originating from the University of Swansea, were kindly provided by Dr. J. Asquith (Toxicol Laboratories Limited, Ledbury). Cells were received at an unknown passage number and used in experiments for up to 10 passages after receipt.

The maintenance of more than one cell line in the same laboratory presents a problem of cross contamination (Paul, 1975). Great care was therefore taken to maintain the purity of cultures throughout these studies. Simultaneous handling of different cell lines was never undertaken and bottles of medium were designated for use with only one particular cell line.

2.8 Cell Culture Methods

2.8.1 Maintenance of Cell Lines

The cell lines employed in these studies are all capable of monolayer growth attached to glass surfaces. Stock cultures were routinely maintained in culture medium in 150 ml clear, soda-glass 'medical' flat bottles at 37.5°C. For some experiments monolayer cultures were maintained in 25 cm^2 growth area plastic T/C flasks.

Cultures were never allowed to reach a point where cells were released from a confluent monolayer, since this can lead to an increased heterogeneity within the cell population (Ham and Puck, 1962). Routine subculture protocols were designed to keep cells in a state of active growth, whilst never allowing them to reach complete confluence (2.8.4).

2.8.2 Preparation of cell suspensions from monolayer cultures

The essential procedure in the maintenance of cells in culture is subculture, involving the transfer of cells from one culture vessel to another. To effect this for monolayer cultures a suspension of cells must first be obtained. Once in suspension cells can also be greatly diluted and plated so that colonies arise from single cells, a procedure important in many experiments.

The same method was used for the preparation of cell suspensions of all the three cell lines employed, although the trypsin solutions, incubation and centrifugation conditions were varied (Table 2.2).

Prior to manipulation cultures of cells in glass bottles were examined by naked eye. For routine subculture the culture medium had to be clear, with no floating cellular debris, and not unduly acidic or basic, as indicated by the phenol red component. Cell growth was required to be greater than 50% confluent. Where cultures were grown

Table 2.2 Trypsin solutions, incubation and centrifugation conditions for subculturing Chinese hamster cell lines maintained in different culture media.

CELL LINE	CULTURE MEDIUM	TRYPSIN SOLUTION	INCUBATION TIME AND TEMPERATURE	CENTRIFUGATION TIME AND SPEED
CHO-K1A	F10 EMEM EMEM+LC	0.05%	5 min 37.5°C	5 min 1500 rpm
	199 199+LC	0.05%	4 min 37.5°C	5 min 1500 rpm
V79-379A	F10	0.025%	3 min 37.5°C	3 min 1000 rpm
	EMEM EMEM+LC	0.025% with 0.02% EDTA	30 sec 20°C	3 min 1000 rpm
CHO(S)	F10	0.05%	5 min 37.5°C	5 min 1500 rpm

in plastic T/C flasks closer examination of cell growth, under phase contrast microscopy, was possible. For these cultures cells had to have a normal appearance, dependent on cell type, with no refractile granules in their cytoplasm. If a particular culture failed to fulfil these requirements it was discarded.

The medium was removed from the culture and the monolayer rinsed with 5 ml of trypsin solution (Table 2.2). This was discarded and replaced by another 2 ml of trypsin solution. The culture was incubated, as indicated (Table 2.2) and then shaken vigorously to release the cells. The suspension of cells was aspirated with a sterile Pasteur pipette, added to 5 ml of serum containing medium in a T/C tube, and sedimented by centrifugation, as indicated (Table 2.2). The supernatant was discarded and the cells resuspended in 5-10 ml of medium, at an approximate cell density of 10^6 cells ml^{-1}. Although the Ca^{++} and Mg^{++} present in culture medium and serum provide some protection against the deleterious effects of the enzyme, resuspending the cells in trypsin-free medium limits its carry over into fresh cultures. This was found to be of particular importance in the maintenance of V79-379A cells, and CHO-K1A cells grown in 199 medium.

2.8.3 Determination of cell density

This was achieved by haemocytometer count, a method which also allows visual examination of the cells prior to experiments. Cells were thoroughly mixed before a small volume was withdrawn from just below the surface of the suspension, and introduced into the haemocytometer chamber. A total cell count was performed on eight large squares of the haemocytometer grid under phase contrast microscopy. If cell clumping was observed the count was discarded, and the suspension aspirated to break up the clumps and new sample taken.

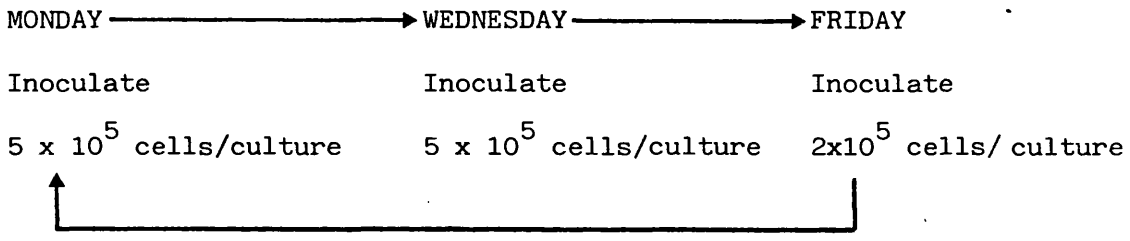
If the cell density was greater than 150 per large square, at which point counting becomes impeded by crowding, the suspension was further diluted and sampling repeated.

Cell count per ml^{-1} is given by:

cell $ml^{-1} = 10^4$ n, where n is the average no. of cells per large square.

2.8.4 Subculture Routine

To maintain cultures in a state of active growth the cells were subcultured at low inoculation density at least three times a week, in the following protocol.



Aliquots of counted cell suspensions were added to 14.5 ml volumes of culture medium, pre-warmed to 37.5°C, in standard culture bottles and the cells evenly distributed by gentle agitation. Culture bottles were then purged with 5% CO_2 in air mixture for 10-15 sec (2.2.4), capped tightly and returned to the incubator. As photo-products toxic to mammalian cells are known to be formed from certain media components (Wang et al., 1974), cultures were incubated in the dark. For the maximum surface area to be available for growth, bottles were incubated horizontally.

For use in experiments, cultures were put up at the subculture preceding the experiment. When a culture was required for use 3 days later 2 x 10^5 cells were inoculated, and for use 2 days later 5 x 10^5 cells were inoculated.

2.8.5 Cell Storage

Stocks of cells were routinely stored frozen in liquid N_2, or its overlying vapour, in the presence of a cryoprotectant. Either 5% DMSO or 8% glycerol were employed as cryoprotectants. No noticeable differences in subsequent recovery of growing cells was observed with either of these compounds.

A culture in a state of active growth and ready for routine subculture was selected. A cell suspension was prepared (2.8.2) and the cell density determined (2.8.3). A number of cells was then withdrawn and adjusted to 5 x 10^5 cells ml^{-1}, first with medium and finally the required volume of cryoprotectant. This cell suspension was thoroughly mixed and 1 ml volumes immediately distributed into 2 ml screw-capped polypropylene ampoules. Once distributed into ampoules the cells could be kept at 4°C for many hours without any noticeable effects on recovery. Batches of 8 ampoules were cooled at 1°C min^{-1} in the freezing unit on top of a LR-33-10 liquid N_2 freezer, to below -70°C, and then rapidly transferred to a liquid N_2 freezer for long-term storage. A few days after every freezing operation one ampoule was removed from storage. If the cells could be recovered to normal growth the freezing procedure was considered successful.

2.8.6 Recovery of Cells from Storage

After removal of the ampoule from storage, the contents were thawed rapidly at 37°C and the ampoule swabbed with 70% alcohol. The

cells were transferred by sterile Pasteur pipette to 14 ml of prewarmed culture medium in a culture bottle and evenly distributed by gentle agitation. The culture bottle was purged with 5% CO_2 in air mixture and incubated at 37.5°C. Within 2-3 hours the cells had usually attached to the glass surface, and after 2-3 days were grown sufficiently to be passaged. The culture was then subjected to the normal subculture routine.

2.8.7 Dilution Plating

For the majority of experiments the plating of a small number of single cells into petri dishes, and their subsequent growth into macroscopic colonies is a key operation. This single-cell plating technique allows the determination of the plating (cloning) efficiency, the proportion of cells in a given population which are viable, measured as their ability for clonal growth.

Although the dilution plating procedure varies with the nature of the particular experiment the following is the basis for all the procedures. 4.9 ml aliquots of culture medium in 50 mm T/C petri dishes were equilibrated at 37.5°C in a humidified 5%CO_2 atmosphere, in plastic incubation boxes. To prevent cell loss through attachment to glass surfaces, which occurs rapidly at 37.5°C (Ham and Puck, 1962), all other medium and solutions were used at room temperature. A cell suspension was prepared (2.8.2) and the cell density determined (2.8.3). The counted cell suspension was serially diluted to a final cell density of 10^3 to 2 x 10^4 viable cells ml^{-1}. For each step of the dilution 0.5 ml of the cell suspension was diluted with 4.5 ml of medium without FCS in a T/C tube, and whirlimixed for 3-4 sec. Mammalian cells tend to settle rapidly from suspension because of their large size. To maintain the uniformity of the cell suspensions

the dilution procedure was performed as quickly as possible with frequent mixing. Replicate 0.1 ml volumes of the final dilution were added to the equilibrated T/C dishes and the cells evenly distributed by gentle agitation. Inoculated dishes were replaced in the incubation boxes and provided with a 5% CO_2 atmosphere (2.2.4). The sealed boxes were incubated in the dark at 37.5°C for 7-8 days, a time sufficient for the clonal growth of all three cell lines employed. During incubation the dishes were left undisturbed to prevent the formation of satellite colonies.

2.8.8 Staining

To determine the plating efficiency of the cells, the medium was poured from the culture and the dishes flooded with 0.5% methylene blue in 50% methanol. After 30 min the dishes were carefully rinsed under running tap water and allowed to dry. Colonies were scored by naked eye against a white background, with aggregates containing 50 or more cells qualifying as survivors. Colonies of marginal size were examined under a binocular dissecting microscope to confirm that they fulfilled this criterion. For the majority of experiments, and all those comprising control and test treatments, plates were coded and randomised prior to counting.

2.8.9 Determination of growth parameters

One of the characteristics of Chinese hamster cell lines that made them useful to these studies is their ability to grow under various culture conditions. To ensure that differences in culture conditions would not significantly affect the interpretation of later results growth parameters were determined for the three cell lines, as follows.

Inocula of 2 x 10^5 cells were subcultured with 5 ml of the appropriate culture medium in a number of 25 cm^2 T/C flasks and incubated at 37.5°C. At intervals a culture was removed and its total cell number determined. The medium in the culture flask was withdrawn and placed on a 16 mm graduated round bottomed test tube (MSE Scientific Instruments). The cell monolayer was rinsed with 2 ml of the appropriate trypsin solution (Table 2.2) and dispersed by incubation with 1 ml of trypsin solution. Both the rinse solution and the suspended cells were added to the medium in the tube. The flask was then rinsed with two 1 ml portions of PBS to remove the residual cells. Complete removal of the cells was confirmed by microscopic examination. Cell losses due to adherence was minimised by the use of a single siliconised Pasteur pipette (2.2.5) for all the above transferences. Cells were sedimented by centrifugation (1500 rpm; 5 min) and resuspended in approximately 0.5 ml of the supernatant. The suspension was adjusted to an expected cell density of 2.5 x 10^5 - 1.5 x 10^6 cells ml^{-1} with PBS and the final volume of suspension noted. The cell density was determined by haemocytometer count and the total cell number calculated.

Under all the conditions of growth examined all three cells lines exhibited "classical" growth kinetics, with three distinct phases of growth: i) a lag phase, ii) an exponential growth phase, and iii) a stationary phase. Fig. 2.1 shows a typical growth curve for CHO-K1A cells grown in F10 medium. From such curves two parameters can be determined, the lag time (a), the time taken for growth to commence, and the population doubling time (T). The exponential phase of growth can be represented by the following equation

$$N_t = N_o e^{kt}$$

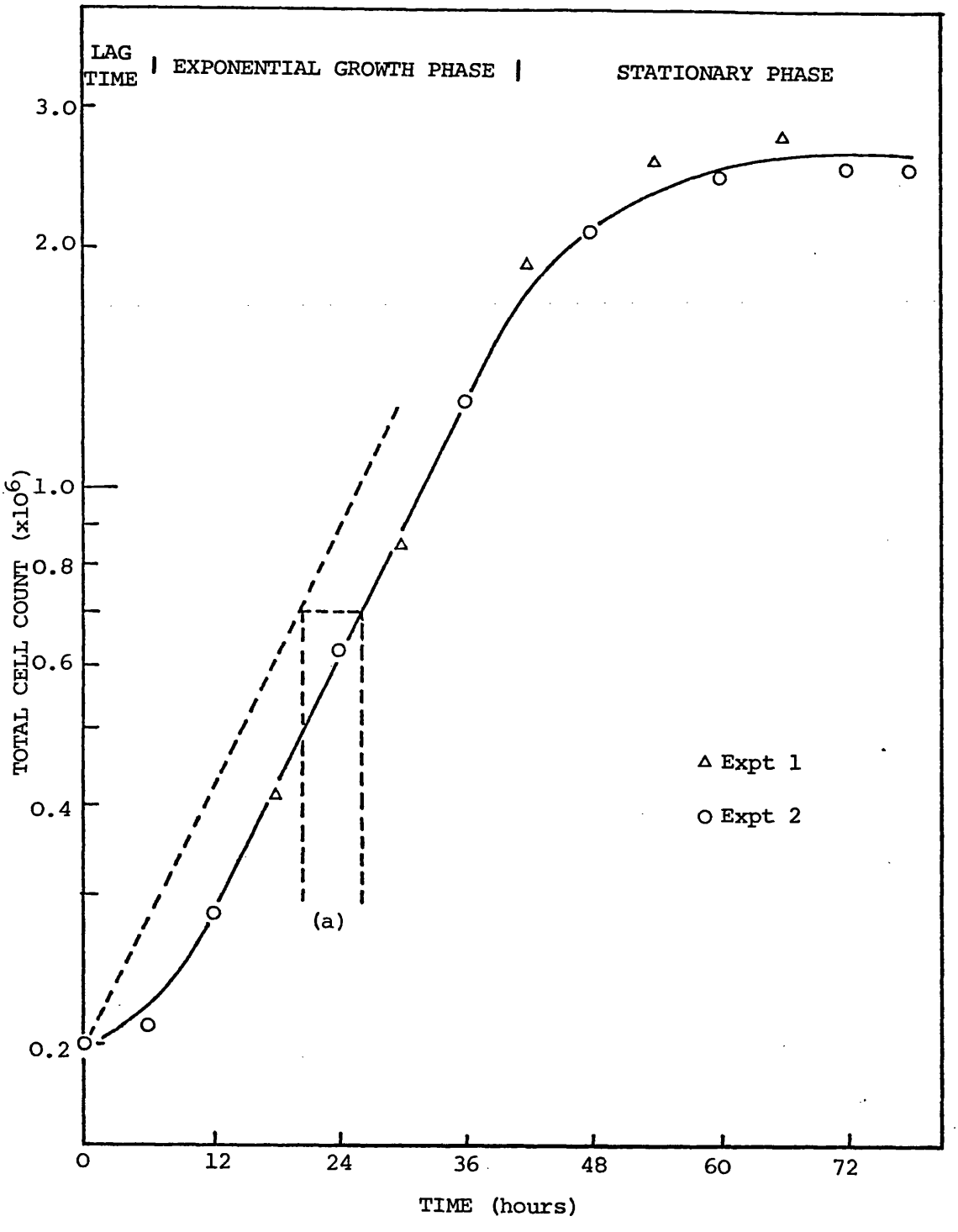


Fig. 2.1 Growth curve for CHO-K1A cells grown in F10.

or in its logarithmic form

$$\log N_t = \log N_o + kt \log 2$$

where N_o is the cell number at time 0

N_t is the cell number at time t

and k is the exponential growth rate constant.

The population doubling time (T) is the inverse of k, i.e. $T = 1/k$. The growth parameters calculated for the three cell lines grown under different culture conditions are given in Table 2.3.

With the exception of CHO-K1A cells grown in the 199 based media, these values are in good agreement with the literature values for various CHO and V79 cell lines (T = 10-16 hr, Bradley et al., 1981; Hsie et al., 1981). The growth parameters for CHO-K1A cells grown in F10 medium, calculated from Fig. 2.1, are in close agreement with those obtained during their initial characterisation (T = 11-12 hr, lag time 6-7 hr, Dewdney, 1982). The slightly longer doubling times for cells grown in the 199-based media might be due to the presence of growth inhibitory factors in the medium. This medium was designed for the short term culture of chick embryo cells, rather than established cell lines, and contains a number of components not present in either F10 or EMEM, most particularly the surface active agent Tween 80 (Morgan et al., 1950).

2.8.10 Effects of TPA on growth parameters of CHO-K1A cells

During these proposed investigations CHO-K1A cells would be exposed to TPA for prolonged periods. Because of its profound effects on cellular growth in cells in culture (see 1.5) the effects of TPA (1 μg/ml) on the growth parameters of CHO-K1A cells was examined.

Table 2.3 Growth parameters for Chinese hamster cell lines maintained in different culture media.

CELL LINE	CULTURE MEDIUM	LAG TIME (hr)	k VALUE (hr^{-1})	DOUBLING TIME (hr)
CHO-K1A	F10	6.0	0.090	11.1
	EMEM	4.8	0.068	14.6
	EMEM+LC	5.4	0.067	14.9
	199	3.0	0.060	16.6
	199+LC	2.5	0.053	18.9
V79-379A	F10	8.5	0.069	14.4
	EMEM	8.0	0.072	13.8
	EMEM+LC	7.5	0.085	11.8
CHO(S)	F10	6.5	0.095	10.5

2×10^5 CHO-K1A cells were inoculated into 25 cm^2 T/C flasks with 5 ml F10 containing either TPA (1 µg/ml) or TPA vehicle (DMSO 1 µl/ml), and incubated. At intervals a culture was removed and its total cell number determined (see 2.8.9). The total cell number at various times following inoculation for DMSO- and TPA-exposed cultures of CHO-K1A cells are given in Table 2.4, together with the calculated growth parameters.

Table 2.4 Growth parameters for CHO-K1A cells grown in F10 medium containing TPA (1 µg/ml) or DMSO (1 µl/ml).

Time(h)	Total Cell Number ($\times 10^6$)	
	DMSO-treated CHO-K1A	TPA-treated CHO-K1A
0	0.21	0.21
18	0.57	0.51
30	1.15	1.29
42	2.33	2.30
66	2.54	2.59
78	2.57	2.75
t½	11.6 hr	11.1 hr
lag time	3 hr	4 hr

When compared with values obtained previously for growth parameters (Table 2.3) these results show that neither TPA nor DMSO affects the growth of CHO-K1A cells in F10.

When examined under inverted phase microscope, the morphology of the cultures was noticeably different. The cells in DMSO-treated cultures retained their normal epitheloid appearance. The cells in

TPA-treated cultures, however, were irregular in shape with an increased number of cytoplasmic processes. Similar effects of TPA on cellular morphology have been reported for CHO cells (Thompson et al., 1980).

2.9 Statistical tests

Statistical tests of difference between any apparently elevated frequencies of mutation in TPA-treated cell populations and mutation frequencies in corresponding TPA-untreated populations were done by the method of Stevens (1942). This method was previously used to test the statistical significance of any effect of TPA on mutagen-elevated mutation frequency in CHO-K1A (Dewdney, 1982), and V79 cells (Trosko and Chang, 1976). It involves the calculation of an upper probability limit for the control mutation frequency. Any mutation frequency higher than this limit, induced in the presence of TPA, was assumed to be significantly elevated above control level. All tests were one-tailed and carried out at the 5% significance level.

CHAPTER 3. THE EFFECTS OF TPA ON GENE MUTATION IN CHO-K1 CELLS

3.1 Introduction

In previous studies in this Department a mutagenic enhancing activity has been detected for TPA (Dewdney and Soper, 1984). It enhanced chemical-induced Oua^R mutation of CHO-K1 cells, while having no effect on spontaneous mutation to Oua^R or mutagen-induced lethality. This effect, however, was mutagen dependent, being observed with MNNG-induced mutation but not with mutations induced by EMS or UV (Dewdney and Soper, 1984). In these studies, the findings of Trosko et al. (1977) and Lankas et al. (1980), that the extent of enhancement of mutagenesis by TPA was dependent upon the duration of promoter treatment, were also confirmed. This chapter reports experiments conducted to extend these previous studies on CHO-K1 cells and examines in more detail the mutagen specific activity of TPA. A description of the mutagens used in these studies is given in Chapter 1 (see 1.7).

The results of preliminary experiments by Dewdney (1982) also suggested that there were differences in mutation enhancement at different genetic loci (Oua^R and TG^R) Experiments were therefore conducted where TPA enhancement of mutation at four independent genetic loci - Oua^R, TG^R, Emt^R and $MGBG^R$ was assessed. The biochemical basis of mutagen testing at these loci is described below.

3.2 Drug resistance markers

3.2.1 Mutation to ouabain resistance (Oua^R)

In mammalian cells the active transport of sodium and potassium ions is associated with the $Na^+ - K^+ - Mg^{2+}$ -activated ATPase (E.C. 3.6.1.3, hereafter referred to as Na^+K^+ATPase) of the plasma membrane (Baker et al., 1974). The steroid compound ouabain (Oua) which specifically inhibits this enzyme, also inhibits the growth of mammalian cells and is ultimately cytotoxic (Baker et al., 1974; Davies and Parry, 1974). Clones of both established and primary cell lines have been obtained, by selection in Oua containing medium, which have greatly increased resistance to Oua cytotoxicity when compared with wild-type cells (Baker et al., 1974; Davies and Parry, 1974; Cole and Arlett, 1976; Arlett, 1977a).

A number of observations have been confirmed that the occurrence of ouabain resistance (Oua^R) reflects a mutant genotype (Baker et al., 1974; Davies and Parry, 1974; Cole and Arlett, 1976; Arlett, 1977a; Gupta and Siminovitch, 1980).

Oua^R is considered to arise by virtue of gene mutations which alter Na^+K^+ATPase, or an adjacent membrane component, such that the enzyme has a reduced affinity for Oua or gives an altered response to the bound drug. EMS and UV, agents capable of inducing base-pair substitution mutations, are efficient inducers of Oua^R mutants (Baker et al., 1974; Arlett, 1977a). Agents which cause chromosomal-deletion type mutations, such as X-rays, and those inducing frameshift mutations, such as 8-methoxypsoralen, are incapable of enhancing mutations to Oua^R (Arlett, 1977b; Gupta and Siminovitch, 1980). This can be readily explained on the basis that Na^+K^+ATPase activity is an essential function for the survival of the cell and mutations which result in its deletion or inactivation will be lethal. Mutations to

Oua^R are likely, therefore, to be base-pair substitutions resulting in a single amino acid or stable conformational alteration of either Na^+K^+ATPase itself or some adjacent membrane components.

3.2.2 Mutation to 6-thioguanine resistance (TG^R)

The biochemical basis of the resistance to the cytotoxic actions of the purine analogue 6-thioguanine (TG) is well established and has been extensively reviewed by Caskey and Kruh (1979). Briefly, TG is converted into the nucleoside 5'monophosphate, thioguanosine monophosphate, by the enzyme hypoxanthine-guanine-phosphoribosyl-transferase (E.C. 2.4.2.8, hereafter abbreviated to HGPRT), present in the purine salvage pathway. After further phosphorylation this can then be incorporated into nucleic acids in place of guanine. Although sufficiently similar to guanine to be metabolised in an identical manner, TG is sufficiently dissimilar to functionally alter the DNA and RNA into which it is incorporated and thus causes cell death.

Clones of rodent and human cells have been isolated, from primary and established cell lines, which are markedly more resistant to the toxic actions of TG than their parental cell strains (Abbondandolo, 1977). This is almost certainly a consequence of HGPRT deficiency and is confirmed by the observations of greatly reduced or altered HGPRT enzyme activity in cell-free extracts from TG^R clones of both CHO-K1 (Hsie et al., 1975) and human lymphoblastoid cells (Epstein et al., 1977). Additionally, 98% of TG^R CHO-K1 clones will not incorporate ^{3}H-hypoxanthine into their nucleic acids, presumably because they have lost the ability, through loss of HGPRT, to metabolise this exogenous purine (Hsie et al., 1975).

A 'back selection' system has also been utilized to prove that TG^R cells are HGPRT-negative ($HGPRT^-$). Normal ($HGPRT^+$) cells can

survive when grown in the presence of the *de novo* purine synthesis inhibitor aminopterin, as long as they are provided with exogenous hypoxanthine (HX) and thymidine. Under these conditions, $HGPRT^+$ cells are still able to obtain sufficient amounts of purine nucleosides to maintain normal nucleic acid synthesis, through the purine salvage pathway. $HGPRT^-$ cells, however, are unable to utilize HX because this pathway is missing. When plated into hypoxanthine, aminopterin and thymidine containing medium (HAT medium) $HGPRT^-$ cells are growth inhibited and eventually die. Using this, O'Neill et al. (1977) have shown that 98% of TG^R CHO-K1 mutants, are incapable of growth in HAT medium and are presumed to be $HGPRT^-$.

The gene coding for HGPRT is located on the X-chromosome in man (De Mars, 1968) and in the hamster (Caskey and Kruh, 1979). Thus, only one HGPRT locus is present in male cells and only one is expressed in female cells (Albertini and De Mars, 1970). As HGPRT function is coded by a hemizygous gene, TG^R has been shown to behave like a recessive genetic trait in appropriate cell hybrids (Paul, 1975).

There is considerable evidence that a phenotypic change to TG^R represents a true gene mutation (O'Neill et al., 1977, 1979; Hsie, 1980).

All classes of physical and chemical mutagens have been reported to induce mutations to TG^R in CHO-K1 (O'Neill et al., 1977; Hsie, 1980), V79 (Arlett, 1977a, 1977b) and L5178Y mouse lymphoma cells (Cole and Arlett, 1976; Arlett, 1977b). De Mars and Jackson (1977) suggest that TG^R variants can result from all conventional classes of mutations, except large scale or whole chromosome deletions.

3.2.3 Mutation to emetine resistance (Emt^R)

The ipecac alkaloid emetine is a rapidly acting reversible inhibitor of protein synthesis (Grollman, 1966). Its mechanism of action is similar to that of cycloheximide in that it inhibits polypeptide chain elongation. The molecular lesion associated with Emt^R has yet to be identified although it appears to be localized in the 40S ribosomal subunit (Gupta and Siminovitch, 1977).

Clones of HeLa and CHO cells have been isolated, by single-step selection, which are markedly more resistant to the cytotoxic actions of emetine than their parental cell strains (Grollman, 1966; Gupta and Siminovitch, 1976). Protein synthesis in cell-free extracts of Emt^R CHO mutants is resistant to the inhibitory action of emetine, confirming that the molecular lesion in these Emt^R cells lies in the protein synthesis pathway (Gupta and Siminovitch, 1976, 1977).

For CHO cells Emt^R has been shown to be a true gene mutation (Gupta and Siminovitch, 1976). Somatic cell hybridization studies show that Emt^R is phenotypically recessive to Emt^S (Gupta and Siminovitch, 1977).

As the Emt locus codes for an essential gene function only limited classes of mutagens are capable of inducing Emt^R in CHO cells. Base-pair substitution mutagens, such as alkylating agents and UV, are efficient inducers of Emt^R (Gupta and Singh, 1982; Singh and Gupta, 1982). Tritium decay of ^3H-thymidine incorporated into DNA and frameshift mutagens, such as ICR 191, are incapable of enhancing mutation to Emt^R (Gupta and Siminovitch, 1980).

3.2.4 Mutation to methylglyoxal bis(guanylhydrazone) resistance ($MGBG^R$)

Polyamines are ubiquitous molecules which influence many biochemical reactions, particularly those involved with cell proliferation and synthesis of macromolecules. Methylglyoxal bis(guanylhydrazone) (MGBG) is a powerful inhibitor of S-adenosyl-L-methionine decarboxylase (E.C. 4.1.1.50, SAMD), a key-enzyme in polyamine synthesis (Williams-Ashman and Schenone, 1972). MGBG has been used clinically in the treatment of leukaemia because of these antiproliferative properties.

MGBG is extremely toxic to cells both *in vivo* and *in vitro*, although clones of established CHO cells and rat myoblasts have been isolated which are resistant to the actions of MGBG (Mandel and Flintoff, 1978). None of these isolated mutants, however, show any alterations in SAMD activity, and $MGBG^R$ was found to result from a dramatic reduction in polyamine and MGBG transport across the cell membranes (Mandel and Flintoff, 1978).

The stability of the $MGBG^R$ phenotype and its increased frequency after mutagenesis suggests that resistance to MGBG represents a true genetic mutation, although definite proof is still lacking (Mandel and Flintoff, 1978). However, in somatic cell hybrids the $MGBG^R$ phenotype behaves recessively (Mandel and Flintoff, 1978).

As polyamine synthesis is essential for cell viability only limited classes of mutagens induce $MGBG^R$ in CHO cells (Gupta and Singh, 1982). Base-pair substitution mutagens are effective inducers of $MGBG^R$, while frameshift mutagens do not enhance the frequency of $MGBG^R$ (Gupta and Siminovitch, 1980).

3.3 The effects of TPA on chemically-induced mutation of CHO-K1 cells to Oua^R

In CHO-K1A cells, TPA has been shown to enhance the recovery of Oua^R mutants induced by MNNG, but not those induced by EMS (Dewdney and Soper, 1984). The first part of this investigation was to examine the effect of TPA on Oua^R mutations induced by mutagens producing a wide variety of DNA lesions, using a standard experimental procedure. The test mutagens used were MNNG, MNU, MMS, EMS, DMAE, DMAP, CEP and DMBA (see 1.7).

Quantification of the frequency of mutation of CHO-K1A cells to Oua^R was assessed by an *in situ* protocol previously validated for this cell line (Dewdney, 1982). In this type of assay the cells are plated, treated with a mutagen and then left undisturbed except for the addition of the selecting agent. The optimum conditions for the recovery of CHO-K1A Oua^R mutants, as characterised by Dewdney (1982) are as follows: the initial density of the cells on selection plates, 2 x 10^5 cells/90 mm dish/10 ml medium; the mutation expression time, 49 hr; the mutant selection time, was 8 days; and the concentration of Oua in the selection medium, 3 mM.

3.3.1 Toxic response tests: single plated CHO-K1A cells.

As mutation assays are expensive and time consuming, it is desirable to have an indication of the mutagen doses likely to yield meaningful results. For a given mammalian cell line the dose range over which the mutagenic agent produces lethality is usually similar to that over which it produces significant mutations (Kao and Puck, 1969; Carver et al., 1979). Dose-response curves were therefore constructed for each mutagen prior to its use in mutation assays. Since an *in situ* protocol would be used for the assessment of Oua^R,

the toxicity of the mutagens towards single-plated cells was determined.

5 x 10^5 CHO-K1A cells were inoculated into a 150 ml culture bottle with 15 ml F10, gassed with 5% CO_2 and incubated (2.8.4). After two days growth, a cell suspension was prepared (2.8.2) and cell density determined (2.8.4). Aliquots of this cell suspension were serially diluted to final cell densities of 10^3 - $2x10^4$ cells ml^{-1} (2.8.7). Depending upon the expected lethality, replicate 0.1 ml volumes of these suspensions, containing 100-2000 cells, were inoculated into 50 mm T/C dishes containing 4.9 ml F10. If there was uncertainty about the cytotoxicity cells were treated at more than one density. The inoculated plates were incubated for 4 hr, to allow the cells to recover from trypsinisation and to attach. The test concentrations of mutagen or vehicle (2.4) were then added to the culture medium. After a 2 hr mutagen exposure the medium was aspirated and replaced with 5 ml fresh F10. When assessing the cytotoxicity of DMBA the plates were rinsed with two 5 ml aliquots of PBS prior to the addition of fresh medium to remove residual insoluble DMBA. The plates were reincubated undisturbed for 7 days, then stained and the colonies scored 'blind' (2.8.8).

The data obtained are presented as dose-response plots in Figs. 3.1 - 3.3. Relative cell survival (R_i) was calculated as $R_i = Y_i/Y_o$, where Y_i is the plating efficiency after treatment i and Y_o the plating efficiency after treatment o. Plating efficiency is defined as the fraction of cells plated that produce macroscopic colonies.

The dose-response curves for both N-nitroso agents tested, MNNG and MNU, show an immediate simple exponential reduction in cell survival, with an apparent hit number of 1 (Drake, 1970). Below 0.15 relative survival, however, the gradient of these curves decreases.

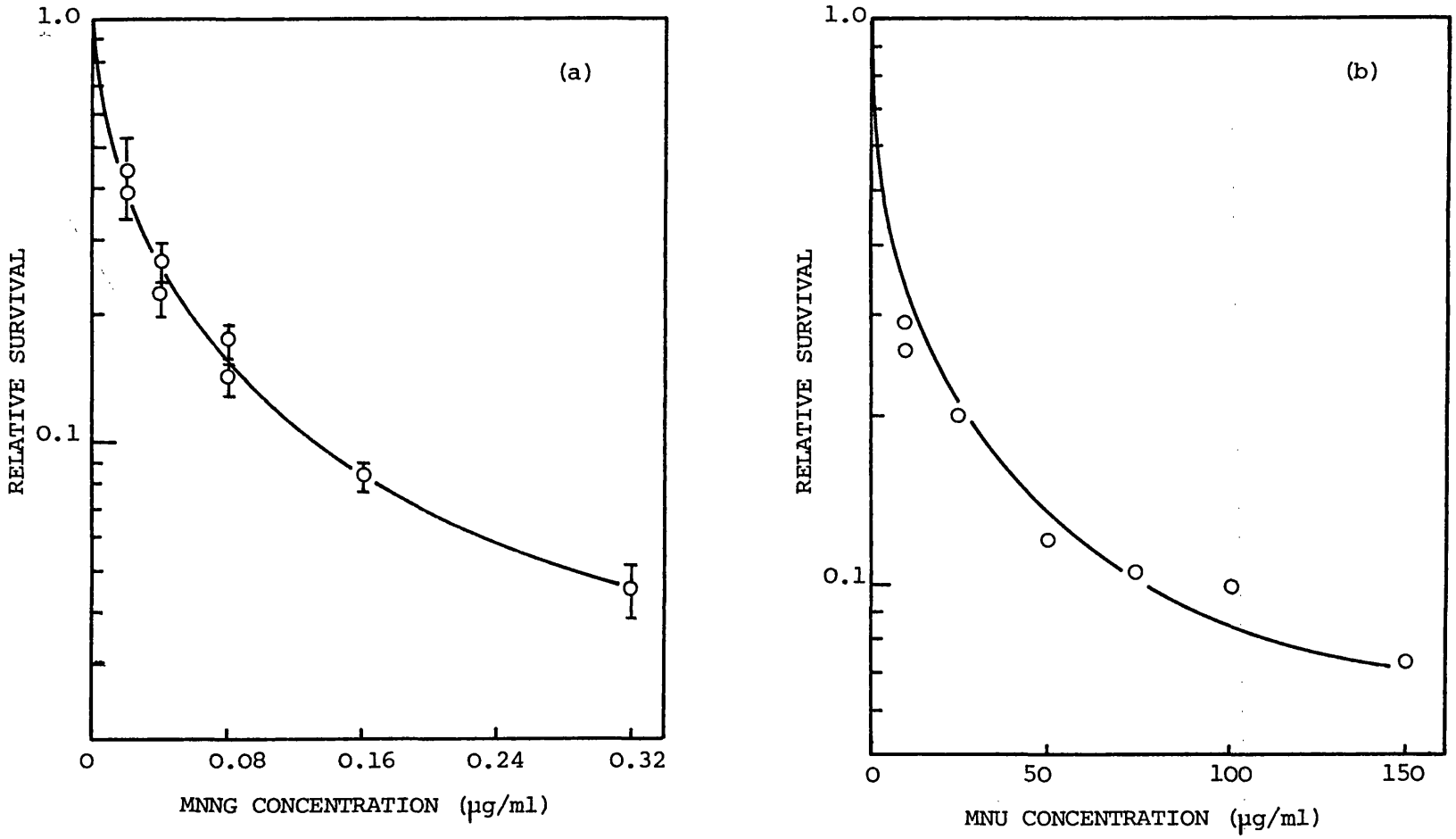


Fig. 3.1 Dose-response curves for single-plated CHO-K1A cells grown in F10 treated with (a) MNNG (b) MNU.

Error bars shown in Fig. 3.1(a) are typical error bars for all survival data.

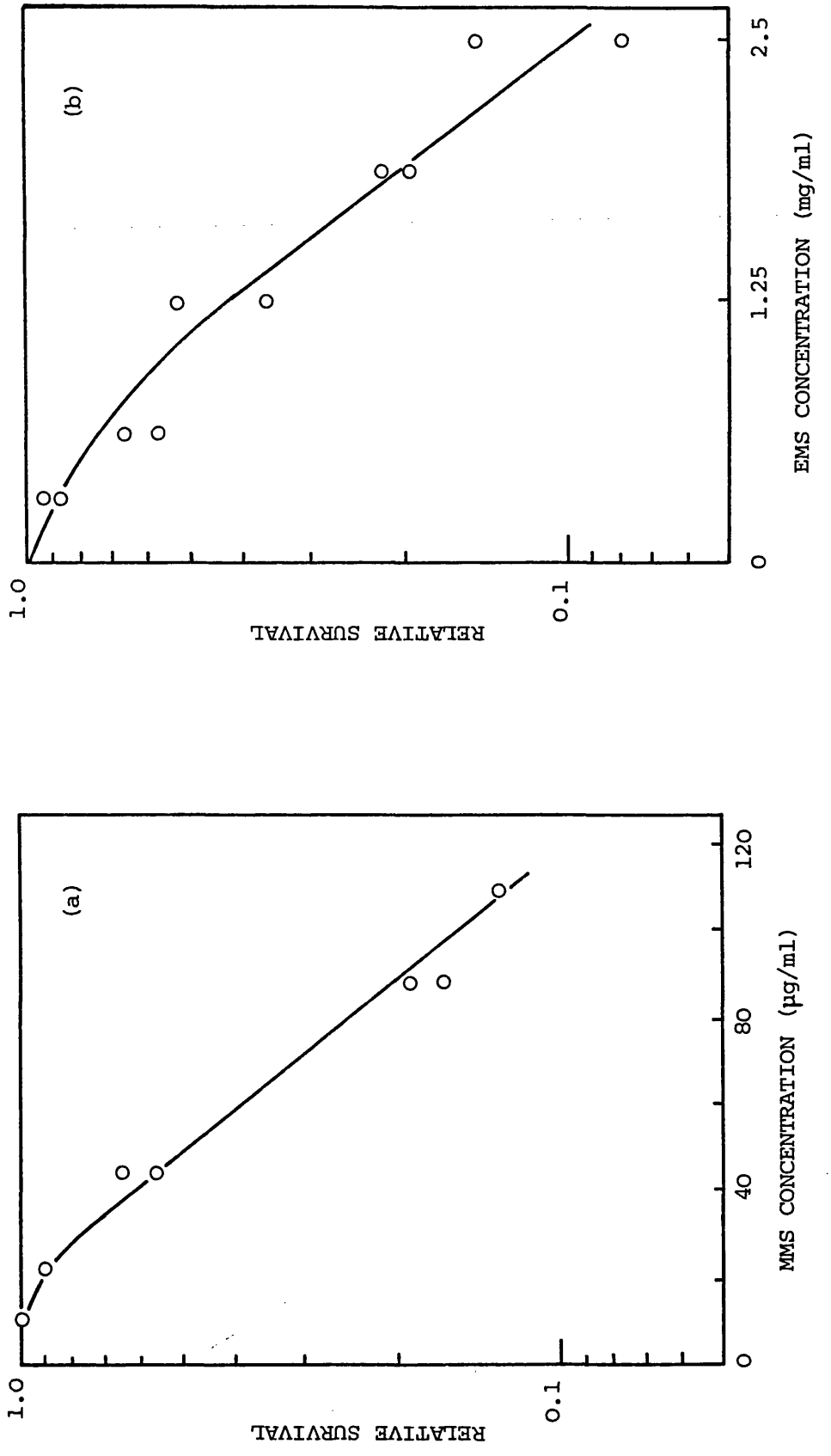


Fig. 3.2 Dose-response curves for single-plated CHO-K1A cells grown in F10 treated with (a) MMS (b) EMS.

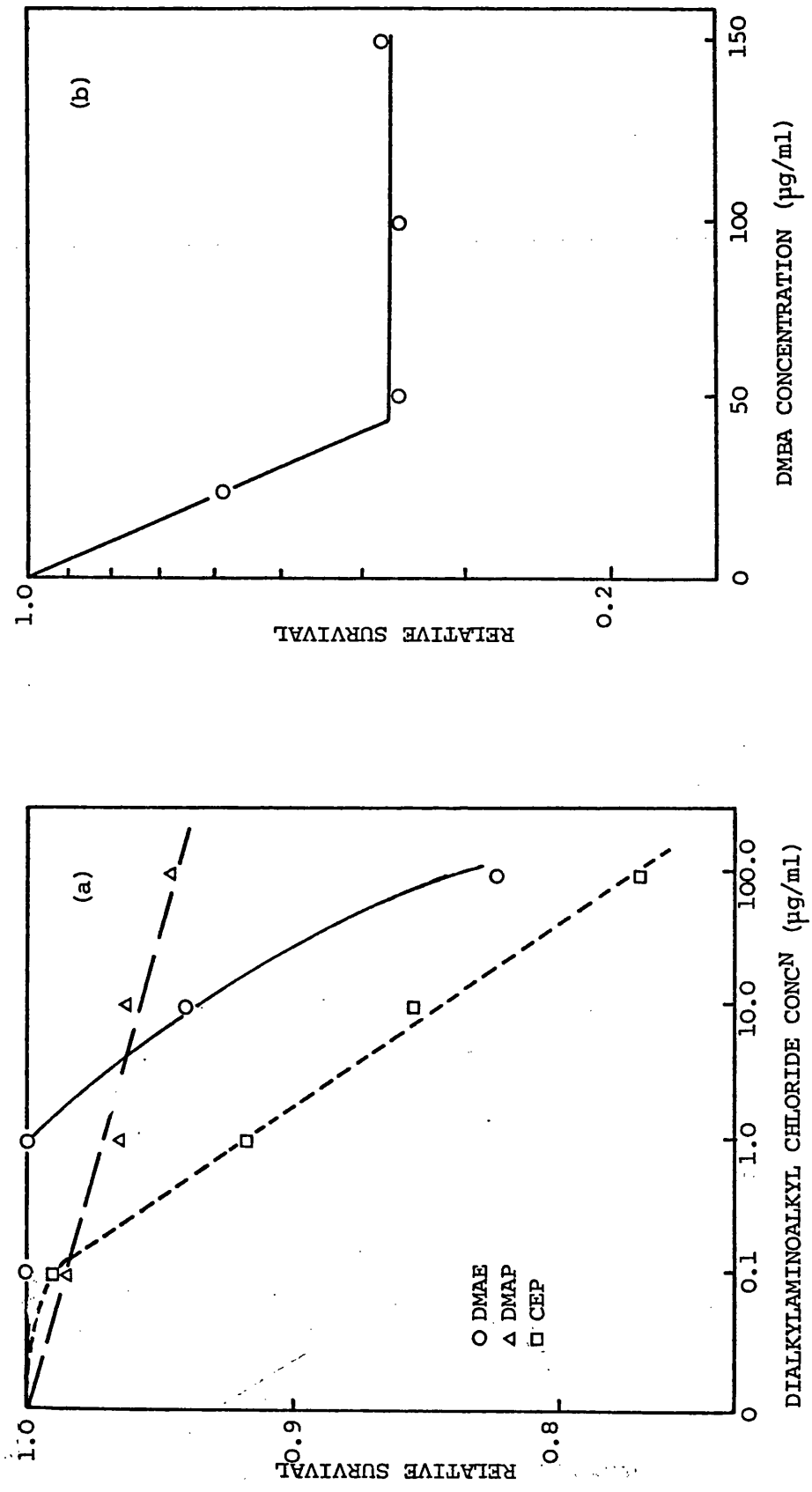


Fig. 3.3 Dose-response curves for single-plated CHO-K1A cells grown in F10 treated with (a) dialkylaminoalkyl chlorides (b) DMBA.

The D_{37} value, defined as the dose required to kill 63% of a cell population and considered to be a valid measure of cytotoxic potency (Carver et al., 1979), can be interpolated for both agents. The D_{37} value for MNNG, from Fig. 3.1a is 0.021 µg/ml. This is comparable with the value of 0.035 µg/ml obtained for CHO-K1A cells during their initial characterisation (Dewdney, 1982). The D_{37} value for MNU, from Fig. 3.1b, is 6.25 µg/ml.

The shouldered-type dose-response curves for CHO-K1A cells treated with MMS (Fig. 3.2a) and EMS (Fig. 3.2b) are of a similar type to those obtained by other workers for CHO-K1 cells (O'Neill et al., 1979; Dewdney, 1982). However, in those studies longer mutagen exposure times (16 hr) and consequently, lower mutagen concentrations were used. No valid comparison of D_{37} values can, therefore, be made. CHO-K1A cells are, however, considerably more resistant to the cytotoxic effects of these agents than to those of the N-nitroso agents.

Only limited data is available on the cytotoxicity of the dialkylaminoalkyl chlorides in mammalian cells. Consistent with their low toxicity in other mammalian cells (Thompson et al., 1981) they are relatively non-toxic to CHO-K1A cells during this short exposure (Fig. 3.3a). A similar rank order of toxicity CEP > DMAE > DMAP, has also been reported in these studies (Thompson et al., 1981).

The toxicity of DMBA was assessed in CHO-K1A cells without the inclusion of an exogenous activating system. However, the dose-response curve (Fig. 3.3b) shows an exponential reduction to a DMBA concentration of 47 µg/ml which suggests that CHO-K1 cells retain some endogenous metabolic activity for this agent. CHO cells have also been reported to retain their metabolic activating capacity (Gupta and Singh, 1982).

3.3.2 Initial investigation of the effects of TPA on chemically-induced Oua^R mutations in CHO-K1A cells.

In these initial experiments, where possible, the concentrations of each mutagen tested was chosen to yield a plating efficiency of approximately 25%. This was the survival level used in TPA mutagenesis enhancing studies in V79 cells where different mutagens were employed (Lankas et al., 1977, 1980).

TPA enhancement of MNNG-induced mutation of CHO-K1A to Oua^R required the promoter to be added immediately at the end of mutagen treatment (2-3 hr), and to be present throughout the remaining mutation expression and the mutant selection periods (Dewdney and Soper, 1984). For this reason, a short mutagen treatment of 2 hr was used during these investigations. The cells were then exposed to TPA, which was maintained in the culture medium for the remainder of the experiment.

TPA was tested at a single concentration of 1 µg/ml, which was maximally effective in previously reported investigations of its possible mutagenesis enhancing activity. The effects of TPA on the recovery of spontaneous Oua^R CHO-K1A mutants was also examined. Thus, for each mutagen four treatment groups were included - control, TPA alone, mutagen alone and mutagen + TPA.

5×10^5 CHO-K1A cells were inoculated into 150 ml culture bottles with 15 ml F10, gassed with 5% CO_2 and incubated (2.8.4). After 48 hr growth a cell suspension was prepared (2.8.2) pooling cells from duplicate cultures where necessary and the cell density determined (2.8.3). This cell suspension was used to seed F10 with approximately 2×10^4 cells ml^{-1}. 10 ml volumes of this seeded medium were inoculated into 90 mm T/C dishes (selection plates). A further portion of the original cell suspension was diluted and used to

inoculate replicate 50 mm T/C dishes, containing 4.9 ml F10, with 200 - 1000 cells, depending on the expected cytotoxicity of the mutagen (survival plates). The inoculated plates were incubated for 4 hr to allow for recovery from trypsinisation and for cell attachment, then the mutagen or vehicle was added to the culture medium. After a 2 hr mutagen exposure, the medium in all plates was replaced with fresh F10. As in the toxicity studies, when DMBA was tested as the initiating mutagen the plates were rinsed with two washes of PBS before the addition of fresh F10. TPA or TPA vehicle (DMSO 1 µl/ml medium) was then added to all plates. Following a further 47 hr incubation, the medium in selection plates was replaced with 10 ml F10 containing 3 mM Oua (3 mM Oua F10) and that in survival plates with fresh F10. TPA or TPA vehicle was added as before. The plates were incubated then stained and scored 'blind' after 6 days (survival plates) or 8 days (selection plates). A diagram summarising this experimental protocol is given in Fig. 3.4.

Using data obtained from such experiments the mutation frequency for a particular treatment can be calculated as the number of mutants per 10^6 survivors using the formula:-

Observed mutation frequency =

$$\frac{\text{total number of mutants} \times 10^6}{\text{total number of cells plated in selection plates} \times \text{plating efficiency of cells in parallel survival plates}}$$

For each mutagen, the number of cells plated and the mutant colonies recovered, the plating efficiencies and the mutation frequencies are given in Tables 3.1 - 3.6.

In all the experiments TPA was not toxic to control cells and did not elevate the frequency of spontaneous Oua^R mutations in CHO-K1A

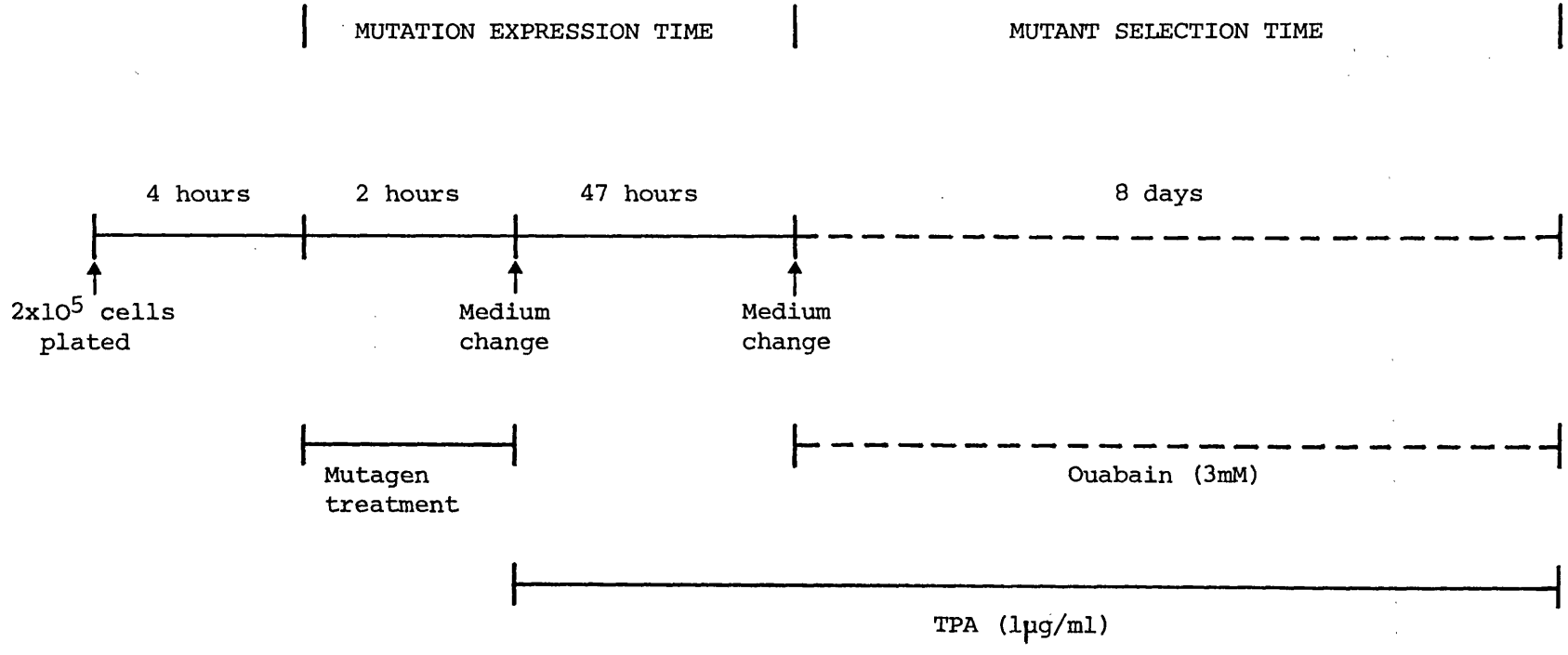


Fig 3.4 Diagrammatic representation of the protocol for the assessment of the effect of TPA on chemical-induced mutation of CHO-K1A cells to Oua^R.

Table 3.1 The effect of TPA(1μg/ml) on the incidence of spontaneous and 0.02μg/ml MNNG-induced Oua^R mutation of CHO-K1A cells grown in F10.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control	72.0	0	0
TPA	69.3	0	0
MNNG	30.3	26	85
MNNG + TPA	31.8	33	103*
Cells/plate - 2.02×10^5			
Plates/treatment - 5			
Expt 2			
Control	74.5	1	1.7
TPA	60.0	1	2.1
MNNG	21.3	12	70
MNNG + TPA	25.5	17	83*
Cells/plate - 2.02×10^5			
Plates/treatment - 4			

* Not significantly higher than MNNG alone (see 2.9). $p > 0.1$

Table 3.2 The effect of TPA(1μg/ml) on the incidence of spontaneous, and 4.4μg/ml MNU-induced Oua^R mutation of CHO-K1A cells grown in F10.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control (a)	83.2	1	1.2
TPA (a)	84.0	2	2.4
MNU (b)	24.8	29	72
MNU + TPA (b)	27.0	33	76*
Cells/plate - 2.02×10^5			
Plates/treatment - (a) 5 (b) 8			
Expt 2			
Control (a)	95.0	1	1
TPA (a)	91.8	1	1
MNU (b)	36.4	26	44
MNU + TPA (b)	35.7	25	50*
Cells/plate - 2.02×10^5			
Plates/treatment - (a) 5 (b) 8			

* Not significantly higher than MNU alone. $p > 0.1$

Table 3.3 The effect of TPA(1μg/ml) on the incidence of spontaneous- and 77μg/ml MMS-induced Oua^R mutation of CHO-K1A cells grown in F10.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control	74.3	1	1.3
TPA	71.8	0	0
MMS	27.9	9	32
MMS + TPA	27.3	9	32
Cells/plate - 2.03×10^5			
Plates/treatment - 5			
Expt 2			
Control	69.0	0	0
TPA	69.2	0	0
MMS	23.3	7	30
MMS + TPA	26.4	8	30
Cells/plate - 2.01×10^5			
Plates/treatment - 5			

Table 3.4 The effect of TPA(1μg/ml) on the incidence of spontaneous and 1.24mg/ml EMS-induced Oua^R mutation of CHO-K1A cells grown in F10.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control	71.4	1	1.4
TPA	69.8	2	2.8
EMS	28.4	46	159
EMS + TPA	26.9	49	179*
Cells/plate - 2.03×10^5			
Plates/treatment - 5			
Expt 2			
Control	70.0	0	0
TPA	76.1	1	1.3
EMS	20.1	44	218
EMS + TPA	22.0	41	185
Cells/plate - 2.01×10^5			
Plates/treatment - 5			

* Not significantly higher than EMS alone. $p>0.1$

Table 3.5 The effect of TPA(1µg/ml) on the incidence of spontaneous and dialkylaminoalkyl chloride-induced Oua^R mutation of CHO-K1A cells grown in F10.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Control	74.0	0	0
TPA	70.3	0	0
DMAE(100µg/ml)	59.4	32	54
DMAE + TPA	54.2	31	57*
DMAP(100µg/ml)	82.5	0	0
DMAP + TPA	75.3	0	0
CEP(100µg/ml)	67.8	19	28
CEP +TPA	63.3	17	27

Cells/plate - 2.01×10^5
Plates/treatment - 5

* Not significantly higher than DMAE alone. $p > 0.1$

Table 3.6 The effect of TPA(1μg/ml) on the incidence of spontaneous and 40μg/ml DMBA-induced Oua^R mutation of CHO-K1A cells grown in F10 without exogenous activation.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Control	57.8	-	-
2%DMSO	40.5	1	2.5
2%DMSO + TPA	41.4	1	2.4
DMBA	17.6	3	17
DMBA + TPA	15.8	3	19*

Cells/plate - $2x10^5$
Plates/treatment - 5

* Not significantly higher than DMBA alone. $p>0.1$

cells. This is consistent with the observations of other workers that TPA is non-mutagenic in the Oua^R system. Additionally, TPA did not enhance the cytotoxic effects of the mutagens.

As has been noted previously EMS (Table 3.4) is a more effective inducer of viable Oua^R mutants than either MNNG (Table 3.1) or MNU (Table 3.2) in CHO-K1A cells (Dewdney, 1982). MMS (Table 3.3), however, is a poor inducer of Oua^R CHO-K1A mutants. A weak mutagenic activity is observed for both CEP and DMAE, while DMAP does not affect the spontaneous levels of Oua^R mutants in CHO-K1A cells (Table 3.5). Although no exogenous activation system was included in this experiment DMBA was still detected as a mutagen, albeit a weak one, at this locus (Table 3.6). This confirms the observations from toxicity studies (Fig. 3.3b) that CHO-K1A cells retain some endogenous metabolic activating capacity for polycyclic hydrocarbons.

The effect of TPA on induced Oua^R mutations in CHO-K1A cells are inconclusive. It may enhance both MNNG- and MNU-induced Oua^R mutations although the mutation frequencies are not significantly higher than following the mutagen alone (Tables 3.1 and 3.2). TPA does not enhance Oua^R mutations induced in CHO-K1A cells by EMS, MMS, the dialkylaminoalkyl chlorides or DMBA (Tables 3.3 - 3.6).

3.3.3 Further investigation of the effects of TPA on Oua^R mutations induced in CHO-K1A cells by alkylating agents.

In V79 cells, the enhancing activity of TPA on both MNNG- and MAM-induced Oua^R mutations increases with increasing mutagen concentration (Lankas et al., 1977). In CHO-K1A cells, TPA has been shown to enhance the frequency of Oua^R mutants induced by 0.05 µg/ml MNNG, but not that induced by 0.04 µg/ml MNNG (Dewdney, 1982). The concentration of a mutagen used as the initiating dose therefore

appears to be important to the effect of TPA on chemical induced gene mutations. The concentrations of the mutagens used in the initial investigation may not have been sufficiently high to elicit a TPA response. The effect of TPA on the Oua^R mutations induced by the simple alkylating agents was therefore re-examined using a higher concentration of each mutagen. In these experiments the concentration of each agent was selected to yield a plating efficiency of approximately 10%.

As in the initial investigation the experimental protocol followed was that indicated by Fig. 3.4.

The results from duplicate experiments for MNNG and MNU are given in Tables 3.7 and 3.8, respectively, and for single experiments for MMS and EMS in Tables 3.9 and 3.10, respectively. Consistent with the results obtained above TPA alone was both non-toxic and non-mutagenic in CHO-K1A cells. Furthermore, it did not enhance the lethal effects of the higher doses of alkylating agents.

Consistent with the results obtained by Dewdney (1982) for this cell line, TPA significantly enhanced the frequency of Oua^R mutants induced by MNNG (Table 3.7), but not that induced by a similarly toxic dose of EMS (Table 3.10). In this extended study of alkylating agents the frequency of Oua^R CHO-K1A mutants induced by a second N-nitroso compound, MNU, is also significantly increased by TPA (Table 3.8). For MMS (Table 3.9), TPA has no effect on the frequency of induced Oua^R mutants.

Table 3.7 The effect of TPA(1μg/ml) on the incidence of spontaneous and 0.08μg/ml MNNG-induced Oua^R mutation of CHO-K1A cells grown in F10.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control	77.8	0	0
TPA	67.8	0	0
MNNG	8.9	12	135
MNNG + TPA	10.9	36	329*
Cells/plate - $2.01x10^5$			
Plates/treatment - 5			
Expt 2			
Control	72.3	1	1.4
TPA	74.0	2	2.7
MNNG	11.2	21	186
MNNG + TPA	11.5	38	328*
Cells/plate - $2.01x10^5$			
Plates/treatment - 5			

* Significantly higher than MNNG alone. $p<0.005$

Table 3.8 The effect of TPA(1µg/ml) on the incidence of spontaneous and 30µg/ml MNU-induced Oua^R mutation of CHO-K1A cells grown in F10.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control	72.5	0	0
TPA	73.0	0	0
MNU	8.8	14	159
MNU + TPA	10.4	41	393*
Cells/plate - 2.01×10^5			
Plates/treatment - 5			
Expt 2			
Control	84.1	1	1.2
TPA	81.7	1	1.2
MNU	8.8	32	364
MNU + TPA	9.5	67	705*
Cells/plate - 2×10^5			
Plates/treatment - 5			

* Significantly higher than MNU alone. $p<0.005$

Table 3.9 The effect of TPA(1μg/ml) on the incidence of 100μg/ml MMS-induced Oua^R mutation of CHO-K1A cells grown in F10.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Control	74.3	-	-
MMS	11.3	6	53
MMS + TPA	10.9	5	46

Cells/plate - 2.01×10^5
Plates/treatment - 5

Table 3.10 The effect of TPA(1ug/ml) on the incidence of 2mg/ml EMS-induced Oua^R mutation of CHO-K1A cells grown in F10.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Control	74.1	-	-
EMS	12.9	29	225
EMS + TPA	10.7	24	224

Cells/plate - 2.01×10^5
Plates/treatment - 5

3.3.4 The effects of adding TPA at different times during mutation expression on the expression of MNNG-induced Oua^{R}mutations in CHO-K1A cells.

Previous studies have reported that the extent of the enhancement of mutagenesis by TPA in CHO-K1A cells was dependent on the duration of promoter treatment (Dewdney and Soper, 1984). TPA enhanced MNNG mutagenesis when added 2 hr following mutagen treatment. However, when added 43 hr or later after MNNG a mutagenesis enhancing activity for TPA was not evident. This time-dependent activity was further investigated by adding TPA to culture medium at different times during mutation expression.

The procedure followed the Oua^R mutagenesis protocol given in Fig. 3.4. CHO-K1A cells were plated and incubated for 4 hr. MNNG (0.08 µg/ml) or MNNG vehicle was then added to the culture medium. At various times following MNNG treatment TPA was added to a set of selection plates and parallel survival plates. 49 hr following the addition of MNNG, medium in selection plates was replaced with 10 ml 3 mM Oua F10 and that in survival plates with 5 ml F10. TPA was added as before. Plates were incubated, stained and scored in the usual way.

The data obtained in two separate experiments for different TPA exposure times are given in Tables 3.11a and b. The mutagenesis enhancing activity of TPA expressed as the enhancement of MNNG-induced mutation frequency is given in Table 3.12. These results show that the mutagenesis enhancing activity of TPA in CHO-K1A cells does vary with the duration of exposure. Mutagenesis enhancing activity reaches a peak when CHO-K1A cells are first exposed 12 hr after MNNG treatment. From 15 hr after MNNG treatment this activity rapidly falls, and when present for mutant selection time only TPA had no effect on the frequency of MNNG-induced Oua^R mutations. This loss of mutagenesis

Table 3.11 The influence of the time of addition of TPA(1μg/ml) on 0.08μg/ml MNNG-induced Oua^R mutation of CHO-K1A cells grown in F10.

TIME OF TPA ADDITION(hr)	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
a) Expt 1			
Control	64.1	0	0
MNNG (t=0)	15.3	10	81
½	18.0	24	165*
1	16.1	21	161*
2	16.8	22	162*
6	16.2	22	168*
12	13.6	24	218*
Cells/plate - 2.02×10^5			
Plates/treatment - 4			
b) Expt 2			
Control	79.8	0	0
MNNG (t=0)	9.0	12	163
½	12.5	34	335*
10	10.5	35	410*
12 (a)	11.1	43	477*
15	11.2	40	440*
18	13.4	25	230
49	10.7	13	150
Cells/plate - 2.03×10^5			
Plates/treatment - 4 (a) 3			

* Significantly higher than MNNG alone.

Table 3.12 The enhancement of 0.08μg/ml MNNG-induced Oua^R mutation of CHO-K1A cells grown in F10 by TPA(1μg/ml) added at different times during mutation expression time.

TIME OF TPA ADDITION (hr)	ENHANCEMENT** FACTOR
½ (a)	2.04*
(b)	2.05*
1 (a)	1.99*
2 (a)	2.00*
6 (a)	2.07*
10 (b)	2.51*
12 (a)	2.69*
(b)	2.92*
15 (b)	2.69*
18 (b)	1.41
49 (b)	0.92

(a) From table 3.11 a)

(b) From table 3.11 b)

* Significant enhancement of MNNG mutagenesis.

** Enhancement factor = $\frac{(\text{Mutation frequency})_{\text{MNNG + TPA}}}{(\text{Mutation frequency})_{\text{MNNG}}}$

enhancing activity when added late in the expression is in agreement with the results of Dewdney and Soper (1984).

3.3.5 The effect of TPA on metabolic cooperation between Oua^R and Oua^S CHO-K1A cells.

The density of cells on plates at the time of mutant selection is an important parameter for *in situ* mutagenesis assays (1.7.1). Cell crowding at this time can lead to an impaired recovery of mutant colonies by metabolic cooperation between normal and mutant cells. TPA is a membrane active compound, and has been shown to enhance the recovery of mutants in TG assay systems by an inhibitory effect on metabolic cooperation (Yotti et al., 1979). A similar effect in the Oua system could explain the observed mutagenesis enhancing activity of TPA in CHO-K1A cells. For this reason the effects of TPA on the recovery of a known number of Oua^R cells cocultured with wild-type (Oua^S) cells was examined in a reconstruction experiment.

a) Isolation of Oua^R CHO-K1A strains:

For the proposed reconstruction study it was first necessary to isolate and propagate strains of Oua^R CHO-K1A cells.

2×10^5 CHO-K1A cells at passage 33 were inoculated into two 90 mm T/C dishes with 10 ml F10 and incubated for 4 hr for cell attachment. MNU (4.4 µg/ml) was then added to the culture medium and the plates reincubated. Mutagen exposure was assumed to be self-limiting as MNU has a reported half-life of approximately 8 min in cell culture medium at 37°C (Jensen et al., 1977). 49 hr after the addition of MNU the medium was aspirated from both plates and replaced with 10 ml 3 mM Oua F10. Plates were incubated for a further 8 days, then one discrete Oua^R colony was located by eye on each. Medium was

aspirated and a sterile glass cylinder (5 mm internal diameter x 10 mm high) whose bottom edge had been coated with sterile silicone grease was placed over each colony. The cylinder was rinsed with a few drops of 0.05% trypsin solution and then a few more drops of trypsin solution were added. After 5 min incubation the cells were suspended by gentle aspiration with a Pasteur pipette and transferred into 5 ml 3 mM Oua F10 in a 25 cm^2 T/C flask. After 7 days growth, each of the Oua^R cultures was trypsinised and transferred with 15 ml 3 mM Oua F10 to a 150 ml culture bottle. After two days incubation, the cultures were trypsinised, suspended at 5 x 10^5 cells ml^{-1} in 3 mM Oua F10 plus 5% DMSO and frozen down in 1 ml volumes (2.8.5). These two independent Oua^R cell strains were designated CHO-K1A Oua 1 and CHO-K1A Oua 2.

b) Reconstruction experiment:

The experimental procedure was analogous to the Oua^R mutagenesis protocol (3.3.2).

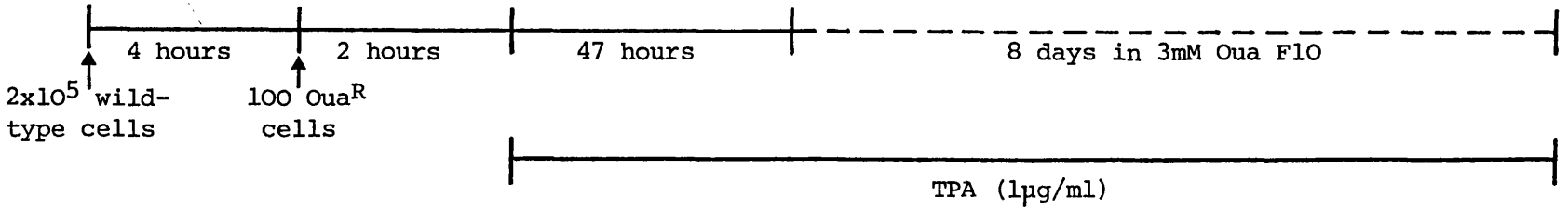
Sets of 90 mm T/C dishes were inoculated with 10 ml volumes of either F10 or F10 containing 2 x 10^5 wild-type CHO-K1A cells, at passage 37 from a 48 hr culture, and incubated for 4 hr. 100 Oua^R cells in 0.1 ml F10 were then added to each plate and distributed by gentle agitation. The Oua^R cells were provided by a 48 hr culture of CHO-K1A Oua 2 cells in 3 mM Oua F10 at passage 37, which was trypsinised and diluted in F10. Plates were incubated for a further 2 hr before TPA* or DMSO was added. Following a 47 hr incubation time, the medium in the plates was replaced with 10 ml 3 mM Oua F10. TPA or DMSO was added as before. The plates were then incubated for a further 8 days, stained and the Oua^R colonies scored 'blind'. Parallel survival plates (unselected controls) of 100 cells in 5 ml F10 in 50

mm T/C dishes were also included for both Oua^R and wild-type CHO-K1A cells.

The numbers of cells plated, the percentage of Oua^R colonies recovered and the plating efficiency of unselected controls are given in Table 3.13, together with a diagram summarising the experimental procedure. These results (Table 3.13) show that in the absence of wild-type cells TPA is not toxic to Oua^R cells. In the presence of wild-type CHO-K1A cells both TPA and DMSO increase the recovery of Oua^R colonies, but to a similar extent. Thus, in this system the mutagenesis enhancing activity of TPA cannot be explained by an inhibition of metabolic cooperation.

*As in previous experiments, TPA was dissolved in DMSO before addition to the culture medium.

Table 3.13 The effect of TPA(1µg/ml) on the recovery of Oua^R CHO-K1A cells in the presence of wild-type Oua^S cells grown in F10.



WILD-TYPE CELLS[a] ($2x10^5$)	OUA^R CELLS[b] (100)	TPA (1µg/ml)	% RECOVERY[c] OF OUA^R
-	+	-	63.0
-	+	+	61.8
+	+	-	81.8
+	+	+	83.0

(a) Plating efficiency Oua^S cells - 95%

(b) Plating efficiency Oua^R cells - 64.8%

(c) Plates/treatment - 5

3.4 The effects of TPA on MNNG-induced mutation of CHO-K1 cells to TG^R

An investigation was undertaken to determine the effect of TPA on the frequency of spontaneous and MNNG-induced mutation to TG^R in CHO-K1A cells, to compare with findings in the Oua^R selection system. Although both MNNG- and MNU-induced mutation to Oua^R was enhanced by TPA, MNNG was chosen as the model mutagen for the TG^R experiments. O'Neill et al. (1977) have reported MNNG to be an efficient inducer of stable TG^R mutants in CHO-K1 cells.

3.4.1 Toxic response test: MNNG toxicity to CHO-K1A cells in monolayer culture.

In the protocol for determining TG^R the cells are seeded 24 hr prior to the addition of the mutagen. Thus, it was necessary to determine the toxicity of MNNG towards monolayer CHO-K1A cultures which are then subcultured and plated for survival 24 hr after mutagen treatment.

The dose range of MNNG investigated, 0.02 µg/ml - 0.2 µg/ml, was suggested by the results of O'Neill et al. (1977) and Dewdney (1982).

5×10^5 CHO-K1A cells were inoculated into a number of 25 cm^2 T/C flasks with 5 ml F10 medium without hypoxanthine (F10(-HX)) (2.3.4). After 24 hr growth the test concentration of MNNG or vehicle was added to the culture medium. Following a 24 hr mutagen exposure the cells were suspended in 1 ml of trypsin solution and transferred with 5 ml F10(-HX) to a 16 mm T/C tube. Cells were sedimented by centrifugation, resuspended in 3 ml F10(-HX) and the cell density determined. Each treated cell population was serially diluted and plated for the determination of survival. Depending on the expected lethality 100 - 2000 cells were inoculated into four replicate 50 mm

T/C dishes with 5 ml F10(-HX). If the likely cytotoxicity was uncertain cells were plated at more than one density. Plates were incubated for 7 days, then stained and scored in the usual way.

The data obtained, from two separate determinations are shown in the dose-response curve given in Fig. 3.5. The D_{37} value is 0.023 µg/ml, which is similar to the value of 0.025 µg/ml estimated from the dose-response curve reported by O'Neill et al. (1977) for CHO-K1-BH_4 cells.

3.4.2 Determination of the expression time for TG^R CHO-K1A mutants

HGPRT is a constitutive enzyme in wild-type cells (see 3.2.2). To allow for the dilution and decay of pre-existing HGPRT in the mutant cells, there will be a delay in the expression of the TG^R phenotype (phenotypic delay). For CHO-K1-BH_4 cells the induced frequency of TG^R mutants is reported to reach a stable maximum at 7-9 days of expression and to be independent of the mutagen concentration (O'Neill et al., 1977; O'Neill and Hsie, 1979). Before the TG^R protocol could be used to investigate the mutagenesis enhancing effects of TPA it was necessary to assess the phenotypic expression time of MNNG-induced TG^R CHO-K1A mutants. This was examined for three concentrations of MNNG.

Quantification of the frequency of mutation to TG^R employed essentially the respreading method of O'Neill et al. (1977). 5×10^5 CHO-K1A cells were inoculated into 25 cm^2 T/C flasks with 5 ml F10 (-HX). After a 24 hr growth period, MNNG or vehicle was added to each culture. 24 hr later the medium was removed and the cells dispersed in 1 ml trypsin solution and transferred with 5 ml F10(-HX) to a 16 mm T/C tube. The cells were sedimented by centrifugation and resuspended in 3 ml F10(-HX). Cell density was determined by haemocytometer count.

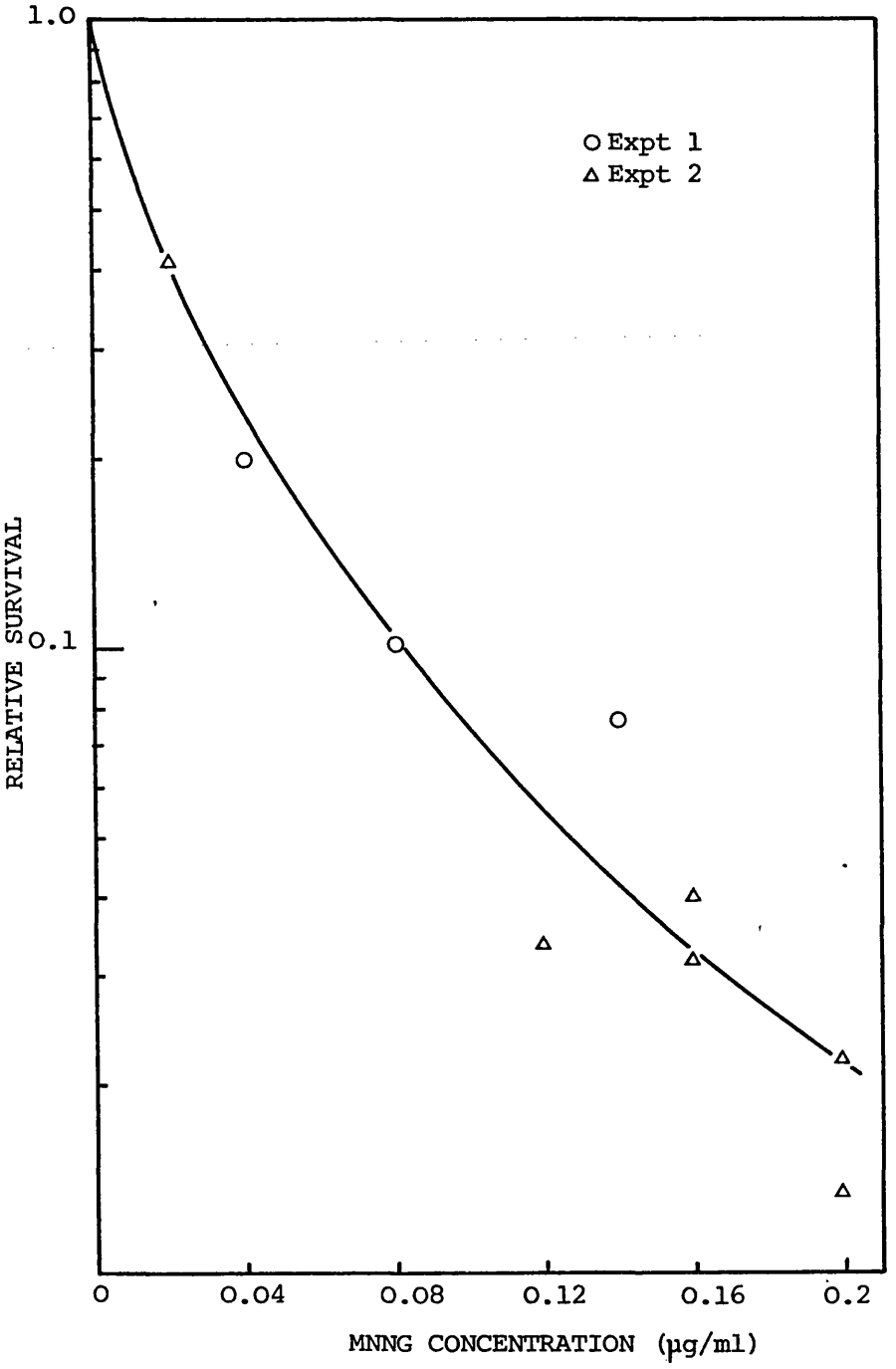


Fig. 3.5 Dose-response curve for CHO-K1A cells grown in F10(-HX) treated with MNNG in monolayer culture.

For determination of initial cell survival, a portion of the cell suspension was diluted and 200 - 1000 cells/5 ml F10(-HX) plated into four 50 mm T/C dishes. For the assessment of mutant induction five 90 mm T/C dishes were each seeded with 1.5 x 10^5 cells in 10 ml of F10(-HX) containing 30 μM TG (2.6.2). From the remaining suspension 10^6 cells were inoculated into a 150 ml culture bottle with 20 ml F10 (-HX). This culture was then subcultured every 48 hr. At each subculture five x 1.5 x 10^5 cells were plated for mutant selection and parallel survival plates inoculated for the determination of plating efficiency. Mutant selection and survival plates were incubated and stained, in the usual way, after 7 days. The time of mutagen addition was designated day 0. Cells were plated for mutant selection, as described, on day 1 (24 hr after initiation of mutagen treatment) and subsequently on days 3, 5, 7, 9, 11 and 15.

The initial cell survivals and the spontaneous and MNNG-induced frequencies of TG^R mutants at each subculture are given in Table 3.14. The induced mutation frequencies are plotted as a function of expression time in Fig. 3.6. The mutation frequency/expression time profile (Fig. 3.6) shows that for all MNNG concentrations TG^R colonies are observed at day 5 of expression. The mutation frequency reaches a peak between 7 and 11 days of expression and is dependent on the MNNG concentration. The maximum frequency of TG^R mutants recovered also increases in a dose-dependent manner.

3.4.3 Initial investigation of the effects of TPA on MNNG-induced TG^R mutations in CHO-K1A cells.

The MNNG concentration used in these studies was 0.08 μg/ml. This yields approximately 10% relative survival, a level similar to

Table 3.14 Frequencies of mutation to TG^R induced in CHO-K1A cells by different concentrations of MNNG. Mutants selected in 30μM TG F10(-HX) after different expression times.

MNNG Conc^n (μg/ml)	INITIAL RELATIVE SURVIVAL	MUTANTS/10^6 SURVIVORS DAY 1	3	5	7	9	11	15
0	1.00(a)	2	0	0	0	0	0	0
0.04	0.15	27	0	257	397	264	222	292
0.08	0.14	15	0	616	1124	1463	1338	967
0.16	0.07	0	355	894	1258	1692	2124	1214

(a) Control plating efficiency - 65.3%

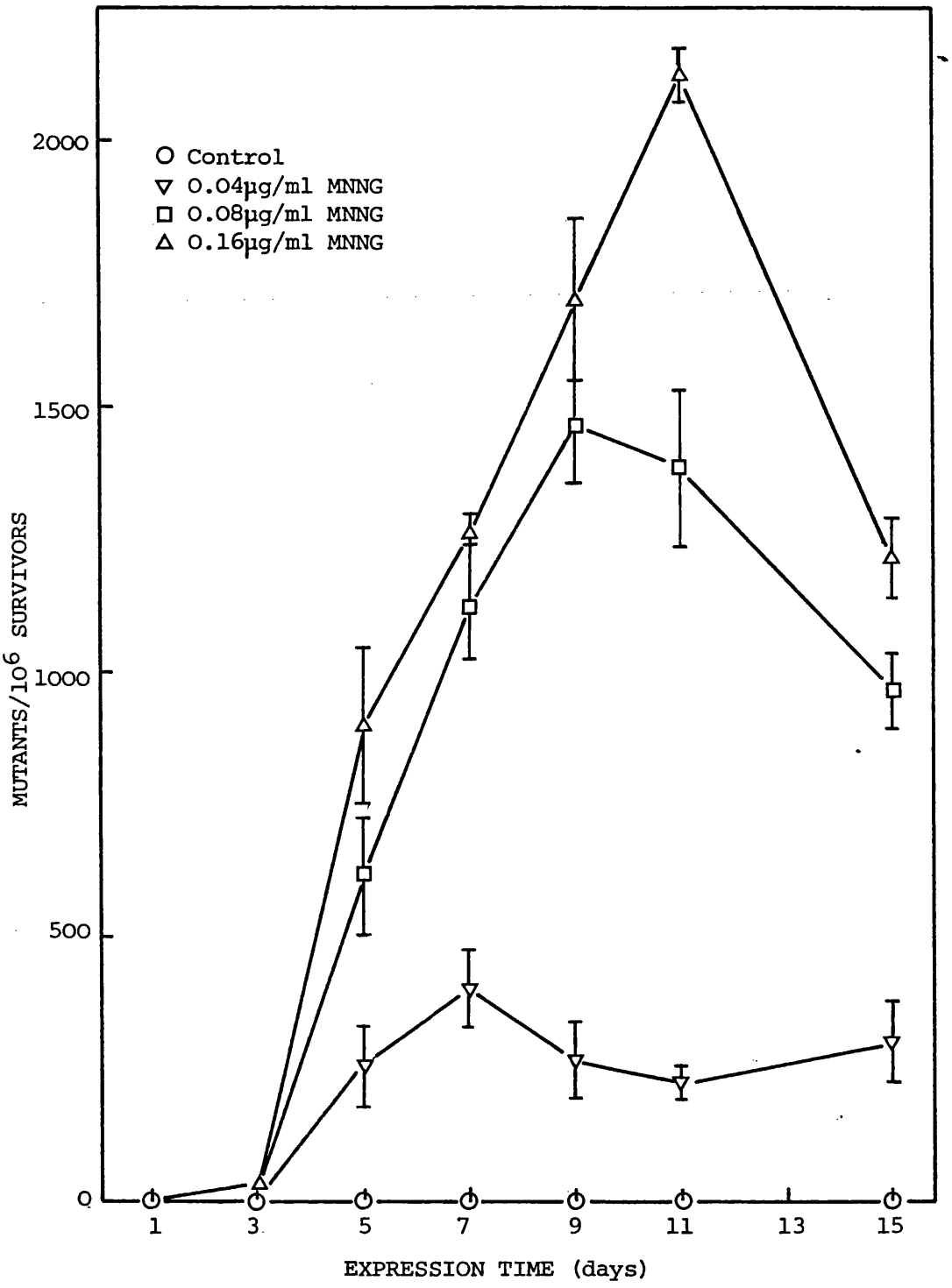


Fig. 3.6 Expression times for TG^R CHO-K1A mutants selected in 30µM TG F10(-HX) induced by different concentration of MNNG.

that used in the Oua^R studies. The effects of TPA on the spontaneous level of TG^R CHO-K1A was also examined. Four treatment groups were thus included - control, TPA, MNNG and MNNG + TPA.

CHO-K1A cells were inoculated into four 25 cm^2 T/C flasks with F10(-HX). After 24 hr growth the cultures were treated with MNNG (0.08 μg/ml) or vehicle and processed as described above (3.4.2). TPA or TPA vehicle was added to the culture medium at the first subculture of the cell population and renewed at each subculture. The tumour promoter or vehicle was added to all mutant selection and survival plates. Thus, TPA was present throughout the mutation expression and mutant selection periods.

As the maximal mutation induction for this concentration of MNNG is not reached until day 9 of expression the cells were first plated into TG containing medium 7 days after mutagen treatment and subsequently on days 9 and 11.

Initial cell survivals and mutation frequencies at the different expression times for each treatment are given in Table 3.15. The spontaneous levels of TG^R mutants recovered in this experiment are considerably higher than those previously observed. TPA does not affect this elevated level of spontaneous mutation to TG^R. For MNNG alone, a peaked profile of mutant recovery, similar to that shown in Fig. 3.6, was observed. TPA has no effect on the frequency of TG^R mutants induced by MNNG. However, in this limited study TPA does appear to affect the shape of the mutation frequency/expression time profile. The previously observed 'peaked' profile, with maximal TG^R recovery at day 9, is replaced by a plateau of induced TG^R mutants recovered between days 7 and 11 for TPA-treated cells. (Figure not shown.)

Table 3.15 The effect of TPA(1μg/ml) on the incidence of spontaneous and 0.08μg/ml MNNG-induced TG^R mutation of CHO-K1A cells selected in 30μM TG F10(-HX) after different expression times. Tumour promoter present from day 1 of expression until the end of mutant selection

TREATMENT	INITIAL RELATIVE SURVIVAL	MUTANTS/10^6 SURVIVORS DAY 7	9	11
Control	1.00(a)	88	56	53
TPA	0.94	139	88	100
MNNG	0.07	1213	1422	1282
MNNG + TPA	0.07	1127	1175	1105

(a) Control plating efficiency - 85.5%

3.4.4 Further investigation of the effects of TPA on MNNG-induced TG^R mutations in CHO-K1A cells.

In cell culture medium at 37°C MNNG has a reported half-life of 14 mins (Jensen et al., 1977). O'Neill et al. (1977) have found that MNNG is only effective as a mutagen for 2 hr after its addition to the culture medium. In the previous experiment TPA was added to cells 24 hr after the addition of MNNG and thus the period of growth during which the cells are susceptible to the mutagenesis enhancing actions of TPA may have already passed. The effects of continuous TPA exposure from the end of effective mutagen treatment, ie 2 hr, were therefore investigated.

In an attempt to reduce the numbers of spontaneous TG^R mutants present, CHO-K1A cells were grown in HAT medium (F10 containing 10^{-6} M aminopterin) for the passage interval immediately prior to their use in this experiment (see 3.3.2). These cells were inoculated into four 25 cm^2 T/C flasks with 5 ml F10(-HX). Cultures were incubated for 24 hr, then treated with MNNG (0.08 µg/ml) or vehicle, and processed as described in 3.4.2. TPA or vehicle was added to the cultures 2 hr after the initiation of MNNG treatment and renewed at each subculture of the cell population. The tumour promoter or vehicle was added to survival and mutant selection plates. For a complete examination of the effect of TPA on the mutation frequency/expression time profile, cells were plated into selection medium at each subculture from day 1.

Initial cell survivals and mutation frequencies on days 1, 3, 5, 7, 9 and 11 for each treatment are given in Table 3.16. The mutation frequencies plotted as a function of expression time are given in Fig. 3.7. A comparison of the results in Tables 3.15 and 3.16, shows that the growth of CHO-K1A cells in HAT medium reduces the incidence of both spontaneous and MNNG-induced TG^R mutants.

Table 3.16 The effect of TPA(1μg/ml) on the incidence of spontaneous and 0.08μg/ml MNNG-induced TG^R mutation of CHO-K1A cells selected in 30μM TG F10(-HX) after different expression times. Tumour promoter present from 2hr following MNNG treatment until the end of mutant selection.

TREATMENT	INITIAL RELATIVE SURVIVAL	MUTANTS/10^6 SURVIVORS DAY 1	3	5	7	9	11
Control	1.00(a)	41	37	59	34	36	36
TPA	0.78	41	41	37	56	78	34
MNNG	0.19	0	61	323	485	737	672
MNNG + TPA	0.18	0	0	618*	683*	716	762

(a) Control plating efficiency - 69.1%

* Significantly higher than MNNG alone.

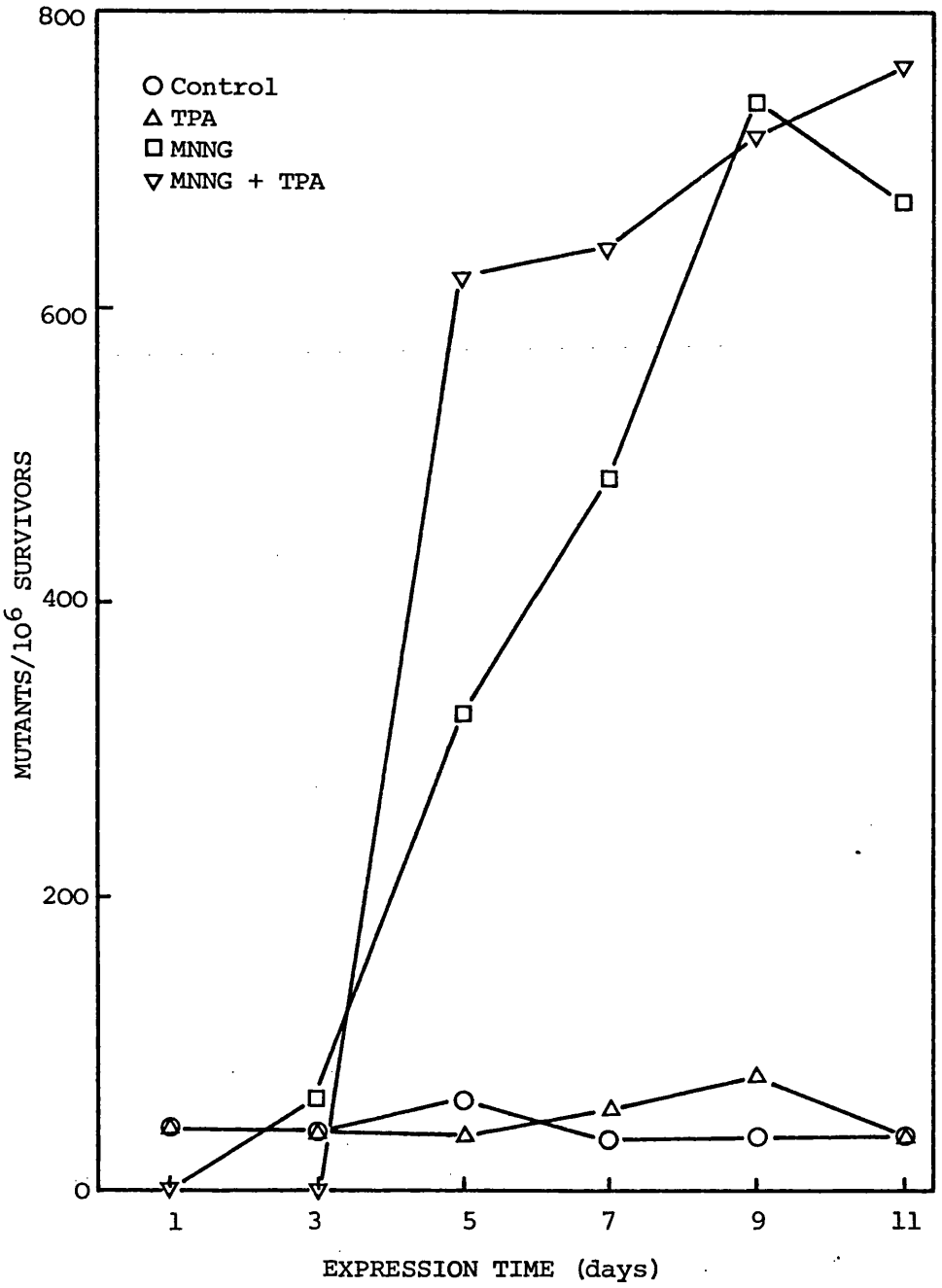


Fig. 3.7 The effect of TPA(1μg/ml) on the recovery of 0.08μg/ml MNNG-induced TG^R CHO-K1A mutants selected in 30μM TG F10(-HX). Tumour promoter present from 2hr following MNNG treatment until the end of mutant selection.

TPA has no effect on either the frequency of spontaneous TG^R mutants or on the maximum frequency of MNNG-induced TG^R mutants. It does, however, alter the expression profile for MNNG-induced TG^R mutants. As shown in Fig. 3.7 continuous exposure to TPA from the end of mutagen treatment decreases the phenotypic delay for TG^R mutants. A significant enhancement of MNNG-induced TG^R mutations is observed on days 5 and 7 of expression. Although not signficantly higher than for MNNG alone on days 9 and 11, the recovery of TG^R mutants is then maintained until day 11 of expression, with no loss of mutants during subsequent subcultures.

3.5 The effects of TPA on MNNG-induced mutation of CHO-K1 cells at other drug resistance markers

Gupta and Singh (1982) have reported a multiple drug-resistance marker mutation assay for CHO cells, in which mutagenic responses can be assessed simultaneously at five independent genetic loci. Oua^R and TG^R studies with TPA have shown that the action of tumour promoters may be loci specific. A multiple marker system may thus provide a more extensive screening system for compounds with tumour promoting activity. The inclusion of other markers, together with TG^R and Oua^R may also provide information as to the identity of the specific genetic lesions enhanced by TPA in these mutagenesis systems. The effect of TPA on MNNG-induced mutation of CHO-K1A cells to resistance at two of these markers, Emt^R and $MGBG^R$ was investigated.

3.5.1 Mutagenic effects of MNNG at the Emt^R and $MGBG^R$ loci in CHO-K1A cells

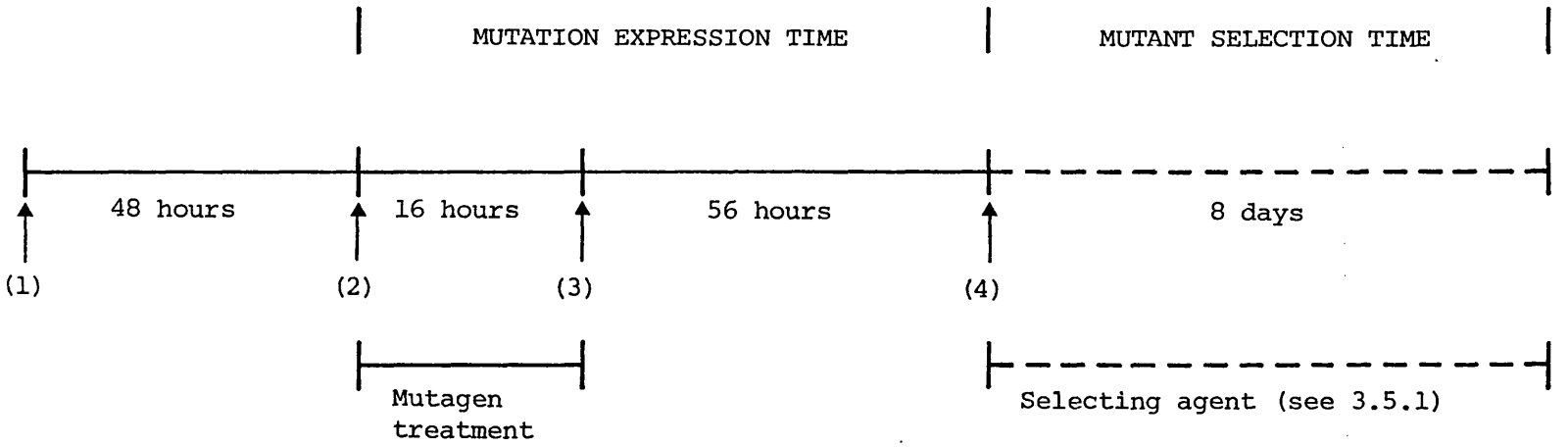
The mutagenic response of MNNG at the Emt^R and $MGBG^R$ markers has not previously been reported, although other alkylating agents are effective inducers of mutations at these loci. The effects of a single MNNG concentration (0.08μg/ml) were investigated using the replating method of Gupta and Singh (1982). Resistance to Oua^R was included in this experiment as a control marker. The concentration of Oua suggested by Gupta and Singh (1982) for use in this system (1.6 mM) was, however, increased to 3 mM, the concentration used in Oua^R studies (3.3).

Four 90 mm T/C dishes were seeded with 4×10^5 CHO-K1A cells in 15 ml F10 and incubated for 48 hr. The medium was then replaced with 15 ml fresh F10 and MNNG (0.08 μg/ml) or vehicle was added to the culture medium of duplicate plates. Following a 16 hr mutagen exposure

the cells were dispersed and pooled separately. Cell density was determined by haemocytometer count. For the determination of initial cell survival, a portion of each suspension was diluted and 100 - 1000 cells/5 ml F10 plated into four 50 mm T/C dishes. The remaining cells were plated into 90 mm T/C dishes at a cell density of 4×10^5 cells/15 ml F10. After a further 56 hr incubation the cells from these plates were dispersed and pooled separately. The cell density was determined and adjusted to 10^6 cells/ml. 1 ml volumes of this suspension were then inoculated into 90 mm T/C dishes with 9 ml F10 containing the desired final concentration of selecting agent - Emt 1.5×10^{-7} M, MGBG 5×10^{-6} M, or Oua 3 mM. Parallel survival plates were also inoculated with 200 cells/5 ml F10 in 50 mm T/C dishes. Mutant selection and survival plates were incubated in the usual way for 7 days (survival plates) or 10 days (selection plates), then stained and scored 'blind'. A diagram summarising this experimental protocol is given in Fig. 3.8.

The initial cell survivals, the number of mutants recovered and the mutation frequencies at the three loci are given in Table 3.17.

The incidence of spontaneous Emt^R and $MGBG^R$ CHO-K1A mutants are comparable with those reported for CHO cells (Gupta and Singh, 1982). MNNG does induce resistant CHO-K1A mutants at the three genetic loci assessed in this experiment. The frequency of MNNG-induced $MGBG^R$ CHO-K1A mutants is similar to that induced by other alkylating agents in CHO cells (Gupta and Singh, 1982). The incidence of MNNG-induced Oua^R CHO-K1A mutants is, however, lower than that previously obtained in this cell line using an *in situ* protocol. Possible reasons for this are discussed in Chapter 7. The incidence of MNNG-induced Emt^R CHO-K1A mutants is higher than that reported for other alkylating agents in CHO cells (Gupta and Singh, 1982). However, for CHO-K1A cells the



(1) Cells seeded - $4x10^5$ cells/90 mm dish/15 ml F10.

(2) Medium change and mutagen added.

(3) Cells replated - $4x10^5$ cells/90 mm dish/15 ml F10.

(4) Mutants selected - 10^6 cells/90 mm dish/10 ml F10.

Fig 3.8 Diagrammatic representation of the experimental protocol for assessing mutagenic effects at the Emt^R and $MGBG^R$ loci in CHO-K1A cells.

Table 3.17 The mutagenic effect of 0.08μg/ml MNNG at three genetic loci in CHO-K1A cells grown in F10.

TREATMENT	INITIAL RELATIVE SURVIVAL	DRUG RESISTANCE MARKER					
		OUA^R		EMT^R		$MGBG^R$	
		TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Control	1.00(a)	0	0	0	0	1	0.4
MNNG	0.14	24	45	24	45	8	14

(a) Control plating efficiency - 77.8%

concentration of Emt (1.5×10^{-7} M) was not sufficient to completely kill wild-type cells within the period of mutant selection. Consequently, it was very difficult to distinguish Emt^R colonies against a background of wild-type cell growth.

3.5.2 Determination of Emt concentration for the selection of Emt^R CHO-K1A mutants.

Preliminary studies showed that the optimum Emt concentration for the selection of CHO-K1A cells was between 1.5×10^{-7} M and 3.0×10^{-7} M. These experiments were performed by seeding 10^6 CHO-K1A cells into 10 ml F10 containing Emt at concentrations between 1.5×10^{-7} M and 12×10^{-7} M in 90 mm T/C dishes. Growth was assessed after 10 days by staining plates in the usual way. 1.5×10^{-7} M Emt allowed wild type cell growth, while at 3×10^{-7} M Emt no growth was visible.

The effects of three concentrations of Emt, 1.5×10^{-7} M, 2.0×10^{-7} M and 2.5×10^{-7} M, on the recovery of MNNG-induced Emt^R CHO-K1A mutants was investigated. 4×10^5 CHO-K1A cells were seeded into 90 mm T/C dishes with 15 ml F10 and then processed as described in 3.5.1. At the end of the mutation expression time 10^6 cells were plated into 90 mm T/C dishes with F10 containing the desired final concentration of Emt. Parallel survival plates were also inoculated. The plates were incubated, stained and scored in the usual way. The data obtained are given in Table 3.18.

Table 3.18 The effect of Emt concentration on the recovery of Emt^R CHO-K1A mutants induced by 0.08 μg/ml MNNG.

Emt concn. ($\times 10^{-7}$ M)	Colonies/plate	Total mutants
1.5	4,2,5,3,7	21*
2.0	5,1,3,4,4	17**
2.5	1,6,3,2,3	15

* considerable wild-type cell growth

**evidence of some wild-type cell growth

It was concluded from this data that a concentration of 2.5×10^{-7} M Emt is sufficient to reduce background wild-type cell growth, without significantly affecting the growth of Emt^R mutants.

3.5.3 The effects of TPA on spontaneous and MNNG-induced mutation of CHO-K1A cells at three genetic loci.

Evidence from other mutation studies has suggested that for a maximal effect the presence of TPA is required from 2 hr after the initiation of MNNG treatment until the end of mutant selection. The effects of this TPA exposure on spontaneous and MNNG-induced mutation of CHO-K1A at the Emt^R, $MGBG^R$ and Oua^R loci was assessed using the protocol described in 3.5.1. TPA or vehicle was added 2 hr after the initiation of MNNG treatment. TPA or vehicle was renewed at each subculture and added to all selection and survival plates. The Emt concentration in selection medium was increased to 2.5×10^{-7} M. As for other mutation studies where the effects of TPA on spontaneous gene mutations were also assessed 4 treatment groups were included - control, TPA alone, MNNG alone and MNNG + TPA.

The initial survivals, the numbers of mutants recovered and the mutation frequencies for each treatment at each resistance marker are given in Table 3.19. Consistent with the observations from other resistance marker systems, these results show that TPA has no effect on the frequency of spontaneous mutants recovered for any of the three genetic loci examined. TPA, also, does not effect the recovery of $MGBG^R$ CHO-K1A mutants induced by MNNG.

The results for the recovery of Oua^R CHO-K1A mutants suggest that TPA may exert a cytotoxic effect on newly arisen Oua^R cells. This may be the result of additional stresses placed on the membranes of cells aready known to be sensitive to trypsinisation (Arlett, 1977a).

A significant increase in the recovery of MNNG-induced Emt^R CHO-K1A mutants is observed following continuous exposure to TPA. However, the level of Emt^R mutants induced by MNNG alone in this experiment was considerably lower than that observed previously for the same MNNG concentration (see Table 3.18).

Table 3.19 The effect of TPA(1μg/ml) on the incidence of spontaneous and 0.08μg/ml MNNG-induced mutation of CHO-K1A cells grown in F10 at three genetic loci. Tumour promoter present from 2hr following MNNG treatment until the end of mutant selection.

TREATMENT	INITIAL RELATIVE SURVIVAL	DRUG RESISTANCE MARKER					
		OUA^R		EMT^R		$MGBG^R$	
		TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Control	1.00 (a)	2	0.7	0	0	0	0
TPA	0.91	0	0	0	0	0	0
MNNG	0.15	16	16.7	1	1.1	4	4.2
MNNG + TPA	0.14	1	1.1	12	13.1*	5	5.5

(a) Control plating efficiency - 77.7%

* Significantly higher than MNNG alone.

3.6 Conclusions

From the results reported in this chapter the following can be concluded.

1. TPA alone was neither toxic to CHO-K1A cells nor mutagenic at the loci studied.
2. TPA did not enhance the lethal effects of the mutagens tested.
3. The enhancing activity of TPA in CHO-K1A cells is mutagen specific. TPA enhanced the expression of MNNG- and MNU-induced Oua^R mutations, but not mutations induced by EMS, MMS, dialkylaminoalkyl chlorides or DMBA (3.3.2 and 3.3.3).
4. The enhancement of MNNG and MNU Oua^R mutagenesis by TPA was dependent on the dose of initiating mutagen (3.3.3).
5. The effect of TPA on the recovery of induced Oua^R mutants is not due to an inhibition of metabolic cooperation (3.3.4).
6. At the TG locus TPA does not increase the frequency of MNNG-induced TG^R mutants. It does however, reduce the phenotypic delay for CHO-K1A TG^R mutants.
7. In contrast to the results obtained in an *in situ* Oua^R mutagenesis protocol (3.3), TPA does not enhance the expression of MNNG-induced Oua^R CHO-K1A mutants assessed using a replating protocol (3.5). This may be accounted for by a cytotoxic effect of TPA on newly arisen Oua^R mutants.
8. TPA may enhance the expression of MNNG-induced Emt^R mutations, but does not enhance the expression of MNNG-induced $MGBG^R$ mutations in CHO-K1A cells.
9. The enhancement of MNNG mutagenesis by TPA was dependent upon the duration of the promoter treatment. CHO-K1A cells were only susceptible to the mutagenesis enhancing effects of TPA when exposed to the promoter during the first 15 hr after MNNG

treatment for the remaining expression and selection periods (3.3.4).

These conclusions will be discussed, together with those from other experimental chapters, in Chapter 7.

CHAPTER 4. THE INFLUENCE OF CULTURE CONDITIONS ON MUTAGENESIS ENHANCING ACTIVITY IN CHO-K1 CELLS

4.1 Introduction

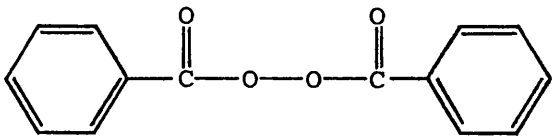
With the growing evidence for the involvement of free radicals in the mechanism of action of TPA (see 1.3 and 1.5), the free radical scavenging potential of culture media has been implicated to explain the discordant results observed for TPA *in vitro* (Emerit and Cerutti, 1981; Nagasawa and Little, 1981). As part of an investigation into its mechanism of action the influence of culture media components on the mutagenesis enhancing activity of TPA in CHO-K1 cells was examined. It has been suggested that TPA itself is not directly involved in either radical release or the subsequent DNA damage. In cultured human lymphocytes DNA damage induced following TPA treatment is mediated by a clastogenic factor, comprised of free arachidonic acid together with low molecular weight peroxides and aldehydes (Emerit and Cerutti, 1981, 1982). Thus, peroxides and aldehydes may be important to the mutagenesis enhancing activity of TPA. For this reason, the influence of culture media components on the activity of benzoyl peroxide (BZP) and formaldehyde (HCHO) as *in vitro* mutagenesis enhancing agents was also investigated.

In contrast to TPA, both BZP and HCHO are highly reactive and may chemically interact with culture media components to produce toxic and mutagenic products. The investigations into the influence of culture conditions on mutagenesis enhancing activity were thus extended to other chemically inert tumour promoters, the linear alkanes.

The biological properties of BZP, HCHO and the linear alkanes are described in the following sections.

4.2 Biological properties of other promoters

4.2.1 Benzoyl peroxide (BZP)



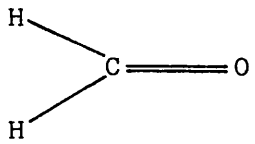
Mol. wt. 242.2

Benzoyl peroxide (BZP) is a free radical generating compound widely used as an additive in cosmetics and pharmaceuticals, particularly those used in the treatment of acne. It can, however, cause irritation when applied to human skin and has come under scrutiny as a human carcinogen. In skin painting studies BZP has been reported to be neither a complete carcinogen (Van Duuren et al., 1963) nor a tumour initiating compound (Slaga et al., 1981). Although when applied to mouse skin BZP does induce hyperplastic and morphological changes similar to those of TPA (Klein-Szanto and Slaga, 1982). A single application of BZP (20 or 40 mg) elicited a marked epidermal hyperplasia noticeable after 48 hr. Epidermal thickness reached a maximum 4 days after BZP application and then decreased to normal values after 10 days (Klein-Szanto and Slaga, 1982). Furthermore, BZP induced a large number of dark keratinocytes, which constituted approximately 10% of the basal cell population 2 to 4 days after treatment. These had also returned to control values 10 days after BZP treatment (Klein-Szanto and Slaga, 1982). When repeatedly applied to mouse skin, following initiation with DMBA, BZP has now been shown to be an effective tumour promoting agent. As little as 1 mg applied twice weekly produced a significant number of papillomas and carcinomas (Slaga et al., 1981).

In vitro, BZP has yet to be tested for a mutagenesis enhancing activity. However, it has been reported to inhibit metabolic cooperation in Chinese hamster V79 cells (Slaga et al., 1981) and human keratinocytes

(Lawrence et al., 1984), in a dose-dependent manner. Following these observations, it has been suggested that the mechanism by which BZP promotes skin tumours may result from membrane changes caused by the free radicals generated by BZP (Slaga et al., 1981).

4.2.2 Formaldehyde (HCHO)



Mol. wt. 30.0

Formaldehyde is a common environmental pollutant found in many occupational settings and in tobacco smoke. It is also a normal biological intermediate, present in all tissues, which participates in many biosynthetic reactions requiring one-carbon units. Endogenous HCHO is, however, reversibly bound to intracellular nucleophiles such as glutathione and tetrahydrofolate.

The reactivity of HCHO towards biological macromolecules has been well documented (Feldman, 1973), and it has been recognised as weakly mutagenic in many mutation assay systems (Auerbach et al., 1977). Single strand breaks induced in DNA by HCHO may be the lesion responsible for its mutagenicity (Fornace et al., 1982). Other investigations, however, have suggested that some of the mutagenic properties of HCHO are caused by reaction with cellular constituents producing altered nucleotides, organic peroxides or free radicals (Auerbach et al., 1977).

HCHO is a respiratory carcinogen in rats, and there is evidence that multiple stages may be involved in HCHO carcinogenesis in this system (Swenberg et al., 1980). This suggests that HCHO might also possess tumour promoting potential. When tested in the two-stage mouse skin model, HCHO is neither a skin tumour initiator nor a complete

skin carcinogen, although it may possess very weak skin tumour promoting activity (Spangler and Ward, 1983).

In vitro HCHO is reported to be a weak promoter of transformation of cells in culture. For MNNG-initiated C3H/10T ½ cells transformed foci were observed following continuous exposure to HCHO (0.5 µg/ml or 1.0 µg/ml) (Frazelle et al., 1983). Neither MNNG nor HCHO treatment alone induced transformed foci.

HCHO can inhibit DNA repair in cells in culture (Grafstrom et al., 1983, 1984) and this might explain its promoting action. In support of this HCHO has been reported to have a mutagenesis enhancing activity *in vitro*. Grafstrom et al. (1985) have shown that HCHO potentiates the mutagenicity of MNU in human fibroblasts.

4.2.3 Linear alkanes

General structure C_nH_{2n+2}

In comparison to BZP and HCHO the linear alkanes are chemically inert compounds. However, they are present in cigarette smoke and human sebum and have been implicated in the enhancement of human carcinogenesis (Bingham and Horton, 1966; Van Duuren and Goldschmidt, 1976).

Members of the series from n-decane to n-hexadecane have been shown to have weak promoting activity, approximately 400-fold less potent than TPA, in mouse skin (Sice, 1966). In this system their promoting activity was related to their carbon chain length.

When assessed *in vitro* for mutagenesis enhancing activity the results obtained are in agreement with *in vivo* studies. At a concentration of 0.12 mM n-decane, n-dodecane and n-tetradecane all enhanced the frequency of MAM-induced mutation of V79 cells to Oua^R, while n-hexane and n-hexadecane were inactive (Lankas et al.,1978).

Similar to their potency in mouse skin, these compounds were about 400-fold less potent than TPA as *in vitro* mutagenesis enhancing agents (Lankas et al., 1978).

The mechanism by which the linear alkanes exert their promoting actions is, as yet, unclear. However, their lipophilic nature is such that they would be expected to partition into cell membranes and they have been reported to affect membrane functions (Horton and McClure, 1971; Horton et al., 1976). Thus, the promoting activity of these compounds may be derived from disturbances of membrane functions.

4.3 The influence of culture conditions on the mutagenesis enhancing activity of TPA in CHO-K1 cells

4.3.1 The influence of L-cysteine on the enhancement of MNNG-induced Oua^R mutations of CHO-K1A cells by TPA.

The sulphydryl compound L-cysteine is reported to react with free radicals and can thus protect cells in culture against the lethal and DNA damaging effects of physical and chemical free radical generating agents (Sasaki and Matsubara, 1977; Raj and Heddle, 1980). The influence of L-cysteine,present in culture media at a concentration of 2×10^{-4} M was therefore investigated on the mutagenesis enhancing activity of TPA in a previously established Oua^R mutation assay (see 3.3). CHO-K1 cells were grown and treated in four culture media EMEM, EMEM + LC, 199 and 199 + LC (see 2.3.4). The cells were grown for at least two passage intervals in the appropriate culture medium prior to each experiment.

The concentration of MNNG used throughout was 0.08 µg/ml. In toxicity studies this was found to yield between 15% and 40% plating efficiency of CHO-K1A cells grown in the different culture media.

For each experiment, the procedure followed the Oua^R mutagenesis protocol described in 3.3.2 and illustratedin Fig. 3.4. CHO-K1A cells were plated into mutant selection and survival plates and incubated for 4 hr. MNNG (0.08 µg/ml) or MNNG vehicle was then added to culture medium. In these, and all subsequent experiments in which MNNG was employed, the mutagen treatment was assumed to be self-limiting (see 3.4.3) and a terminating medium change was unnecessary. 2 hr after the addition of MNNG, TPA or TPA vehicle was added to each plate. 47 hr later the medium in selection plates was replaced with 10 ml medium containing 3 mM Oua and that in survival plates with 5 ml fresh medium. TPA or vehicle was added as before. Plates were reincubated,

stained and scored in the usual way.

The data obtained from duplicate experiments for cells in EMEM and EMEM + LC are given in Tables 4.1 and 4.2, respectively, and for single experiments for cells in 199 and 199 + LC in Table 4.3.

In agreement with the results obtained previously in the Oua^R system (3.3),TPA alone was neither toxic nor mutagenic to CHO-K1A cells grown in any of the culture media employed. Also, TPA did not affect the survival of CHO-K1A cells following MNNG treatment.

As observed previously (Table 3.7), TPA does enhance MNNG-induced mutation of CHO-K1A cells to Oua^R. However, the mutagenesis enhancing activty of TPA is only apparent when L-cysteine is present in the culture medium (Tables 4.2 and 4.3b). F10 medium used in previous experiments (3.3) is also a L-cysteine containing medium. In the absence of L-cysteine TPA does not significantly enhance MNNG mutagenesis (Tables 4.1 and 4.3a).

4.3.2 The influence of glutathione (GSH) on the enhancement of MNNG-induced Oua^R mutation of CHO-K1A cells by TPA.

The series of experiments reported in 4.3.1 indicated that L-cysteine can affect the mutagenesis enhancing activity of TPA. The influence of another sulphydryl free radical scavenging compound, glutathione (GSH), on this enhancing activity was examined. GSH was added to EMEM at a concentration of 2×10^{-4} M (see 2.3.4).

As in other MNNG experiments, the procedure followed that illustrated in Fig. 3.4. CHO-K1A cells were grown for at least two passages in EMEM containing 2×10^{-4} M GSH prior to use in experiments.

The data obtained for duplicate experiments are given in Table 4.4. TPA alone was neither toxic nor mutagenic to CHO-K1A cells, and

Table 4.1 The effect of TPA(1μg/ml) on the incidence of spontaneous and 0.08μg/ml MNNG-induced Oua^R mutation of CHO-K1A cells grown in EMEM.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control	92.5	1	1
TPA	84.5	3	3.4
MNNG	25.6	37	139
MNNG + TPA	37.3	57	148*
Cells/plate - 2.07×10^5			
Plates/treatment - 5			
Expt 2			
Control	77.8	1	2
TPA	74.3	1	2
MNNG	32.3	54	256
MNNG + TPA	32.5	70	330*
Cells/plate - 1.3×10^5			
Plates/treatment - 5			

* Not significantly higher than MNNG alone. Expt 1 $p > 0.1$
Expt 2 $0.1 > p > 0.05$

Table 4.2 The effect of TPA(1μg/ml) on the incidence of spontaneous and 0.08μg/ml MNNG-induced Oua^R mutation of CHO-K1A cells grown in EMEM+LC.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control	73.3	0	0
TPA	68.5	1	1.8
MNNG	30.5	38	153
MNNG + TPA	28.3	60	260*
Cells/plate - 2.04×10^5			
Plates/treatment - 4			
Expt 2			
Control	68.5	1	1.8
TPA	65.8	1	1.9
MNNG	31.8	19	74
MNNG + TPA	32.3	38	146*
Cells/plate - 2.01×10^5			
Plates/treatment - 4			

* Significantly higher than MNNG alone. $p < 0.005$

Table 4.3 The effect of TPA(1μg/ml) on the incidence of spontaneous and 0.08μg/ml MNNG-induced Oua^R mutation of CHO-K1A cells grown in a) 199 or b) 199+LC.

a) 199

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Control	61.4	1	1.6
TPA	66.3	0	0
MNNG	24.0	6	25
MNNG + TPA	24.0	6	25

Cells/plate - 2.01×10^5
Plates/treatment - 5

b) 199+LC

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Control	54.1	2	4.4
TPA	50.2	0	0
MNNG	23.3	7	36
MNNG + TPA	21.3	13	72*

Cells/plate - 1.7×10^5
Plates/treatment - 5

* Significantly higher than MNNG alone. $p < 0.005$

Table 4.4 The effect of TPA(1μg/ml) on the incidence of spontaneous and 0.08μg/ml MNNG-induced Oua^R mutation of CHO-K1A cells grown in EMEM with $2x10^{-4}$M GSH.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control	89.5	0	0
TPA	84.8	2	2.4
MNNG	44.9	103	229
MNNG + TPA	52.3	117	224
Cells/plate - $2x10^5$			
Plates/treatment - 5			
Expt 2			
Control	86.4	-	-
MNNG	43.1	105	244
MNNG + TPA	40.8	117	287*
Cells/plate - $2x10^5$			
Plates/treatment - 5			

* Not significantly higher than MNNG alone. $0.1>p>0.05$

it did not enhance the lethal effects of MNNG. In the presence of GSH, however, the mutagenesis enhancing activity, previously noted for TPA in the presence of L-cysteine, is absent.

4.3.3 The influence of L-cysteine on the enhancement of EMS-induced Oua^R mutation of CHO-K1A cells by TPA.

As culture conditions can affect the enhancing activity of TPA on MNNG-induced mutation of CHO-K1A cells, they may also affect the enhancing activity of TPA towards mutations induced by other mutagens. Thus, the effects of TPA on EMS-induced mutation to Oua^R was investigated in CHO-K1A cells grown in EMEM and EMEM + LC.

The Oua^R mutagenesis protocol given in Fig. 3.4 was used. However, in these experiments a terminating medium change was necessary following a 2 hr exposure to 1.24 mg/ml EMS.

The data obtained for single experiments for CHO-K1A cells grown in either EMEM or EMEM + LC are given in Table 4.5. These show that TPA did not affect the survival of CHO-K1A cells following EMS treatment. In agreement with the results obtained previously (Tables 3.4 and 3.10) TPA did not enhance EMS-induced mutation of CHO-K1A cells to Oua^R, irrespective of culture conditions.

Table 4.5 The effect of TPA(1μg/ml) on the incidence of 1.24mg/ml EMS-induced Oua^R mutation of CHO-K1A cells grown in a) EMEM or b) EMEM+LC.

a) EMEM

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Control	93.2	-	-
EMS	18.3	67	358
EMS + TPA	18.5	69	354

Cells/plate - 2.05×10^5
Plates/treatment - 5

b) EMEM+LC

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Control	67.5	-	-
EMS	15.5	121	776
EMS + TPA	15.5	136	872*

Cells/plate - 2.01×10^5
Plates/treatment -5

* Not significantly higher than EMS alone. $p>0.1$

4.4 The influence of culture conditions on the mutagenesis enhancing activity of benzoyl peroxide (BZP) in CHO-K1 cells

4.4.1 Toxicity of BZP to CHO-K1A cells.

As BZP has not been previously tested for mutagenesis enhancing activity, it was necessary to find a suitable non-toxic concentration for use in such studies. The cytotoxicity of BZP was assessed in CHO-K1A cells grown in three culture media - F10, EMEM and EMEM + LC, The experimental protocol employed a double exposure to BZP, similar to that to be used in mutation experiments.

Suspensions of cells were prepared from 48 hr cultures of CHO-K1A cells grown for at least two passage intervals in the appropriate medium. 200 cells were then plated into 50 mm T/C dishes with 5 ml medium. The seeded plates were incubated for 4 hr before BZP or vehicle (acetone 1 µl/ml) was added. Following a further 47 hr incubation, the medium in all plates was replaced and BZP or vehicle added as before. The plates were incubated for a further 5 days, then stained and scored in the usual way.

The relative survival of CHO-K1A cells treated with different concentrations of BZP in each media are shown in Table 4.6.

Following these studies it was decided to use a BZP concentration of 1.5 µg/ml in mutagenesis enhancing investigations.

4.4.2 The effect of BZP on spontaneous and MNNG-induced Oua^R mutation of CHO-K1A cells under different culture conditions.

The effects of BZP (1.5 µg/ml) on spontaneous and MNNG-induced Oua^R mutations was examined in CHO-K1A cells grown in three culture media - F10, EMEM and EMEM + LC. As for previous MNNG experiments a mutagen concentration of 0.08 µg/ml was used. For each experiment the

Table 4.6 The toxicity of BZP to single-plated CHO-K1A cells grown in different culture media.

BZP conc^{n} (μg/ml)	RELATIVE SURVIVAL F10	EMEM	EMEM+LC
0	1.00	1.00	1.00
0.5	0.92	0.96	1.06
1.0	0.99	0.94	0.93
1.5	1.10	1.03	1.01
2.0	0.92	-	1.01
3.0	0.14	0.01	0.23
Control plating efficiency	87.2%	87.1%	76.8%

Oua^R mutagenesis protocol, given in Fig. 3.4, was followed. BZP (1.5 µg/ml) or vehicle was added 2 hr after MNNG treatment, and maintained in the culture medium for the remaining mutation expression time and the whole of the mutant selection time.

The data obtained for duplicate experiments for CHO-K1A cells in each culture media are given in Tables 4.7 - 4.9. In no instance was BZP alone toxic or mutagenic to CHO-K1A cells. Furthermore, it did not enhance the cytotoxic effect of MNNG. BZP did, however, enhance MNNG-induced Oua^R mutations in CHO-K1A cells, but only in the absence of L-cysteine, i.e. when cells are grown in EMEM (Table 4.8). For cells grown in the cysteine containing media F10 (Table 4.7) and EMEM + LC (Table 4.9), BZP did not significantly increase the recovery of MNNG-induced Oua^R CHO-K1A mutants.

4.4.3 The effect of BZP on EMS-induced Oua^R mutations in CHO-K1A cells under different culture conditions.

As the mutagenic enhancing activity of TPA in the CHO-K1/Oua^R system is mutagen specific, the effects of BZP on the Oua^R mutations induced by EMS was investigated. This was assessed in CHO-K1A cells grown in two culture media - EMEM and EMEM + LC.

The Oua^R mutagenesis protocol (Fig. 3.4) used previously was employed. The EMS concentration used was 1.24 mg/ml and as for other EMS studies a terminating medium change was necessary following a 2 hr mutagen exposure.

The data obtained for cells grown in the two culture media are given in Table 4.10. These results show that BZP had a similar effect on EMS-induced Oua^R mutation to that observed for MNNG-induced Oua^R mutations. BZP did enhance the recovery of EMS-induced Oua^R mutations

Table 4.7 The effect of BZP(1.5μg/ml) on the incidence of spontaneous and 0.08μg/ml MNNG-induced Oua^R mutation of CHO-K1A cells grown in F10.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control	87.2	0	0
BZP	88.3	0	0
MNNG	9.7	8	104
MNNG + BZP	10.6	8	94
Cells/plate - 2.02×10^5			
Plates/treatment - 4			
Expt 2			
Control	75.2	0	0
BZP	76.0	1	1.5
MNNG	6.4	8	147
MNNG + BZP	7.3	8	129
Cells/plate - 2.13×10^5			
Plates/treatment - 4			

Table 4.8 The effect of BZP(1.5µg/ml) on the incidence of spontaneous and 0.08µg/ml MNNG-induced Oua^R mutation of CHO-K1A cells grown in EMEM.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control	89.0	3	5
BZP	84.4	3	5.2
MNNG	28.1	24	126
MNNG + BZP	20.6	43	307*
Cells/plate - $1.7x10^5$			
Plates/treatment - 4			
Expt 2			
Control	87.1	0	0
BZP	68.8	1	1.4
MNNG	39.7	29	91
MNNG + BZP	29.0	42	181*
Cells/plate - $2x10^5$			
Plates/treatment - 4			

* Significantly higher than MNNG alone. $p<0.005$

Table 4.9 The effect of BZP(1.5μg/ml) on the incidence of spontaneous and 0.08μg/ml MNNG-induced Oua^R mutation of CHO-K1A cells grown in EMEM+LC.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control	80.0	1	1.6
BZP	90.0	1	1.4
MNNG	31.4	37	146
MNNG + BZP	27.6	40	179*
Cells/plate - 2.02×10^5			
Plates/treatment - 4			
Expt 2			
Control	77.5	0	0
BZP	-	-	-
MNNG	31.7	53	202
MNNG + BZP	28.9	62	259*
Cells/plate - 2.07×10^5			
Plates/treatment - 4			

* Not significantly higher than MNNG alone. Expt 1 $p>0.1$
Expt 2 $0.1>p>0.05$

Table 4.10 The effect of BZP(1.5μg/ml) on the incidence of 1.24mg/ml EMS-induced Oua^R mutation of CHO-K1A cells grown in a) EMEM or b) EMEM+LC.

a) EMEM

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Control	93.2	-	-
EMS	18.3	67	358
EMS + BZP	16.7	103	603*

Cells/plate - 2.05×10^5
Plates/treatment - 5

b) EMEM+LC

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Control	67.5	-	-
EMS	15.5	121	776
EMS + BZP	10.1	95	934**

Cells/plate - 2.01×10^5
Plates/treatment - 5

* Significantly higher than EMS alone. $p<0.005$

** Not significantly higher than EMS alone. $0.1>p>0.05$

in CHO-K1A cells grown in EMEM (Table 4.10a), but did not significantly increase the EMS-induced Oua^R mutations of cells grown in EMEM + LC (Table 4.10b). This is discussed in Chapter 7.

4.5 The influence of culture conditions on the mutagenesis enhancing activity of formaldehyde (HCHO) in CHO-K1 cells

4.5.1 Toxicity of HCHO to CHO-K1A cells.

To find a suitable non-toxic concentration for use in mutagenesis enhancing studies the toxicity of HCHO was assessed in CHO-K1A cells. The range of HCHO tested, 0.6 - 12 µg/ml, was suggested by the data of Grafstrom et al. (1985). The cytotoxicity of HCHO was assessed using the double exposure procedure, previously used for BZP (see 4.4.1), in CHO-K1A cells grown in two culture media - EMEM and EMEM + LC.

The relative survival of CHO-K1A cells treated with different HCHO concentrations in either EMEM or EMEM + LC are given in Table 4.11.

These results indicate that the cytotoxic effects of HCHO are independent of culture conditions. At concentrations greater than 0.6 µ g/ml continuous exposure to HCHO significantly decreased the survival of CHO-K1A cells. It was thus decided to use a HCHO concentration of 0.6 µg/ml in mutagenesis enhancing studies.

4.5.2 The effect of HCHO on spontaneous and MNNG-induced Oua^R mutations of CHO-K1A cells under different culture conditions.

The Oua^R mutagenesis protocol given in Fig. 3.4 was employed with 0.08 µg/ml MNNG as the mutagen. HCHO (0.6 µg/ml) or vehicle was added 2 hr after the MNNG treatment and was maintained in the culture medium for the remaining mutation expression time and the whole of the mutant selection period.

The data obtained in single experiments for CHO-K1A cells grown in either EMEM or EMEM + LC are given in Table 4.12. These results show that at the concentration selected HCHO was not toxic to either control or MNNG-treated CHO-K1A cells. Furthermore, HCHO alone was

Table 4.11 The toxicity of HCHO to single-plated CHO-K1A cells grown in different culture media.

HCHO concn (μg/ml)	RELATIVE SURVIVAL EMEM	EMEM+LC
0	1.00	1.00
0.6	0.87	0.89
1.5	0.45	0.34
3.0	0.02	0.02
6.0	0	0
12.0	0	0
Control plating efficiency	75.9%	78.0%

Table 4.12 The effect of HCHO(0.6µg/ml) on the incidence of spontaneous and 0.08µg/ml MNNG-induced Oua^R mutation of CHO-K1A cells grown in a) EMEM or b) EMEM+LC.

a) EMEM

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Control	87.6	1	1.1
HCHO	87.3	2	2.3
MNNG	41.3	165	397
MNNG + HCHO	27.4	121	439*

Cells/plate - 2.01×10^5
Plates/treatment - 5

b) EMEM+LC

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Control	85.4	0	0
HCHO	76.3	0	0
MNNG	36.1	86	238
MNNG + HCHO	31.0	115	375**

Cells/plate - 2.00×10^5
Plates/treatment - 5

* Not significantly higher than MNNG alone. $p > 0.1$

** Significantly higher than MNNG alone. $p < 0.005$

non-mutagenic to CHO-K1A cells at the Oua^R locus.

With regard to the mutagenesis enhancing activity of HCHO, culture conditions are important. Similar to the effects observed for TPA (4.3), HCHO only enhance MNNG-induced Oua^R mutation of CHO-K1A cells when cysteine was present in the culture medium.

4.6 The influence of culture conditions on the mutagenesis enhancing activity of the linear alkanes in CHO-K1 cells

The effects of culture conditions on the mutagenesis enhancing activities of the linear alkanes was investigated in CHO-K1A cells grown in F10, EMEM and EMEM + LC. In this series of experiments the concentration of the three alkanes, n-decane, n-dodecane and n-tetradecane, was 17 µg/ml, 20 µg/ml and 24 µg/ml, respectively. These non-toxic concentrations have been found to enhance MAM-induced mutation of V79 cells to Oua^R (Lankas et al., 1978).

The mutagenesis enhancing activity of these compounds was assessed using the Oua^R mutagenesis protocol given in Fig. 3.4. The alkanes were added 2 hr after MNNG treatment and maintained in the culture medium for the remaining expression time and the whole of the mutant selection period.

The data obtained for single experiments for each alkane tested in CHO-K1A cells grown in each culture medium are given in Tables 4.13 - 4.15. These results show that at the concentrations tested none of the linear alkanes alone was toxic or mutagenic to CHO-K1A cells. None of the alkanes affected the survival of CHO-K1A cells following MNNG treatment. In contrast to the results obtained in V79 cells, none of the linear alkanes tested enhanced MNNG-induced mutation of CHO-K1A cells to Oua^R.

Culture conditions had little effect on the activity of these compounds. Increased levels of MNNG-induced Oua^R mutants were recovered when CHO-K1A cells were grown in EMEM + LC following treatment with all the alkanes (Tables 4.13c, 4.14c and 4.15c), but in no instance was this level of mutation significantly higher than for MNNG alone.

Table 4.13 The effect of n-decane(17μg/ml) on the incidence of spontaneous and 0.08μg/ml MNNG-induced Oua^R mutation of CHO-K1A cells grown in a) F10 b) EMEM or c) EMEM+LC.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
a) F10			
Control	78.1	3	3.8
n-decane	74.3	1	1.3
MNNG	7.5	22	293
MNNG + n-decane	6.7	13	193
Cells/plate - 2.02×10^5			
Plates/treatment - 5			
b) EMEM			
Control	84.5	1	1.2
n-decane	81.0	1	1.2
MNNG	49.5	61	122
MNNG + n-decane	45.6	33	71
Cells/plate - 2.03×10^5			
Plates/treatment - 5			
c) EMEM+LC			
Control	77.7	0	0
n-decane	77.2	1	1.3
MNNG	30.9	33	105
MNNG + n-decane	28.0	37	130*
Cells/plate - 2.03×10^5			
Plates/treatment - 5			

* Not significantly higher than MNNG alone. $p>0.1$

Table 4.14 The effect of n-dodecane(20μg/ml) on the incidence of spontaneous and 0.08μg/ml MNNG-induced Oua^R mutation of CHO-K1A cells grown in a) F10 b) EMEM or c) EMEM+LC.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
a) F10			
Control	87.3	0	0
n-dodecane	86.8	0	0
MNNG	10.1	17	167
MNNG + n-dodecane	8.4	15	177*
Cells/plate - 2.02×10^5			
Plates/treatment - 5			
b) EMEM			
Control	77.2	2	2.6
n-dodecane	76.0	2	2.6
MNNG	45.9	45	98
MNNG + n-dodecane	39.8	33	103*
Cells/plate - 2.01×10^5			
Plates/treatment - 5			
c) EMEM+LC			
Control	71.5	4	5.6
n-dodecane	70.5	1	1.4
MNNG	18.8	36	191
MNNG + n-dodecane	27.8	66	236*
Cells/plate - 2.01×10^5			
Plates/treatment - 5			

* Not significantly higher than MNNG alone. $p > 0.1$

Table 4.15 The effect of n-tetradecane(24μg/ml) on the incidence of spontaneous and 0.08μg/ml MNNG-induced Oua^R mutation of CHO-K1A cells grown in a) F10 b) EMEM or c) EMEM+LC

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
a) F10			
Control	101.2	0	0
n-tetradecane	104.5	0	0
MNNG	7.4	11	146
MNNG + n-tetradecane	7.4	11	146
Cells/plate - $2.04x10^5$			
Plates/treatment - 5			
b) EMEM			
Control	66.8	0	0
n-tetradecane	62.5	0	0
MNNG	27.1	57	209
MNNG + n-tetradecane	24.8	55	220*
Cells/plate - $2.02x10^5$			
Plates/treatment - 5			
c) EMEM+LC			
Control	71.8	1	1.4
n-tetradecane	68.6	0	0
MNNG	32.7	50	153
MNNG + n-tetradecane	31.6	55	174*
Cells/plate - $2x10^5$			
Plates/treatment - 5			

* Not significantly higher than MNNG alone. $p>0.1$

4.7 Conclusions

The results reported in this chapter can be summarised as follows.

1. At the concentrations tested none of the tumour promoters, TPA, BZP, HCHO or linear alkanes, was cytotoxic or mutagenic in CHO-K1A cells at the Oua^R locus.
2. The mutagenesis enhancing activity of TPA in CHO-K1A cells was mutagen specific. TPA enhanced the expression of MNNG-induced Oua^R mutations (4.3.1) but not those induced by EMS (4.3.3).
3. The enhancement of MNNG mutagenesis by TPA was only observed when L-cysteine was present in the culture medium (4.3.1). This was not a general effect of sulphydryl compounds, in the presence of GSH the mutagenesis enhancing activity of TPA was abolished (4.3.2).
4. The free radical generating compound BZP enhanced the expression of both MNNG-induced (4.4.2) and EMS-induced (4.4.3) Oua^R mutations, but only in the cysteine free medium EMEM.
5. For HCHO, a similar profile of mutagenesis enhancing activity was obtained as for TPA. Enhancement of MNNG mutagenesis by HCHO was only observed when L-cysteine was present in the culture medium (4.5). (Preliminary results.)
6. In contrast to the results reported for V79 cells (Lankas et al., 1978), a mutagenesis enhancing activity could not be detected for the linear alkanes tested in CHO-K1A cells, irrespective of culture conditions

These conclusions will be discussed, together with those from the other experimental chapters, in Chapter 7.

CHAPTER 5. THE EFFECTS OF TPA ON GENE MUTATION IN OTHER CHINESE HAMSTER CELL LINES

5.1 Introduction

In vivo it is becoming increasingly apparent that tumour promoting agents are both species and organ specific (Table1.1). Similar cell line specificity has also been noted for their mutagenesis enhancing activity *in vitro*. TPA enhanced induced mutation in Chinese hamster V79 cells (Lankas et al., 1977, 1980; Trosko et al., 1977) but not mutations induced in either CHO (Thompson et al., 1980) or mouse lymphoma L5178Y cells (Lasne et al., 1980). Furthermore, in CHO-K1 cells the action of TPA appears to be dependent on themutagenic dose and the culture conditions. Different mechanisms may be involved in the mutagenesis enhancing activity of TPA in different cell lines. The effects of TPA on chemical-induced Oua^R mutations was therefore examined in V79 and CHO cells to compare with the results obtained in CHO-K1 cells.

5.2 The effects of TPA on chemical-induced mutation of V79 cells to Oua^R

5.2.1 Toxic response tests: single-plated V79 cells.

Dose-response curves for V79 cells treated with MNNG and EMS were constructed prior to their use in mutation assays. Since an *in situ* protocol would again be used for the assessment of Oua^R, the toxicity of these mutagens towards single-plated cells was determined. MNNG toxicity was assessed in V79 cells grown in three culture media - F10, EMEM and EMEM + LC. EMS toxicity was assessed in cells grown in EMEM + LC.

Initially MNNG concentrations were selected from the survival data for V79 cells presented by other workers (Roberts et al., 1971; Lankas et al., 1977; Peterson et al., 1979). However, in preliminary studies these doses were found to be far too toxic towards V79-379A cells and the doses were reduced by a factor of 10. The MNNG concentrations selected for this study were in the range 0.02 - 0.16 µg/ml.

The EMS concentration used, 0.3 - 1.25 mg/ml, were suggested by Arlett et al. (1975).

The experimental protocol followed that used previously for toxicity studies in CHO-K1A cells (see 3.3.1). A series of 50 mm T/C dishes were inoculated with 100-2000 V79 cells, depending upon the expected lethality. Cells were provided by 48 hr cultures of V79-379A cells, grown in the appropriate culture medium for at least two passage intervals. Seeded plates were incubated for 4 hr to allow cells to recover from trypsinisation, then dosed with mutagen or vehicle. After a 2 hr mutagen exposure, the medium was replaced in all plates. Plates were incubated, then stained and scored in the usual way after 7 days.

The data obtained are presented as dose-response plots in Fig. 5.1 a-c. By interpolation, the D_{37} value for MNNG is 0.013 µg/ml

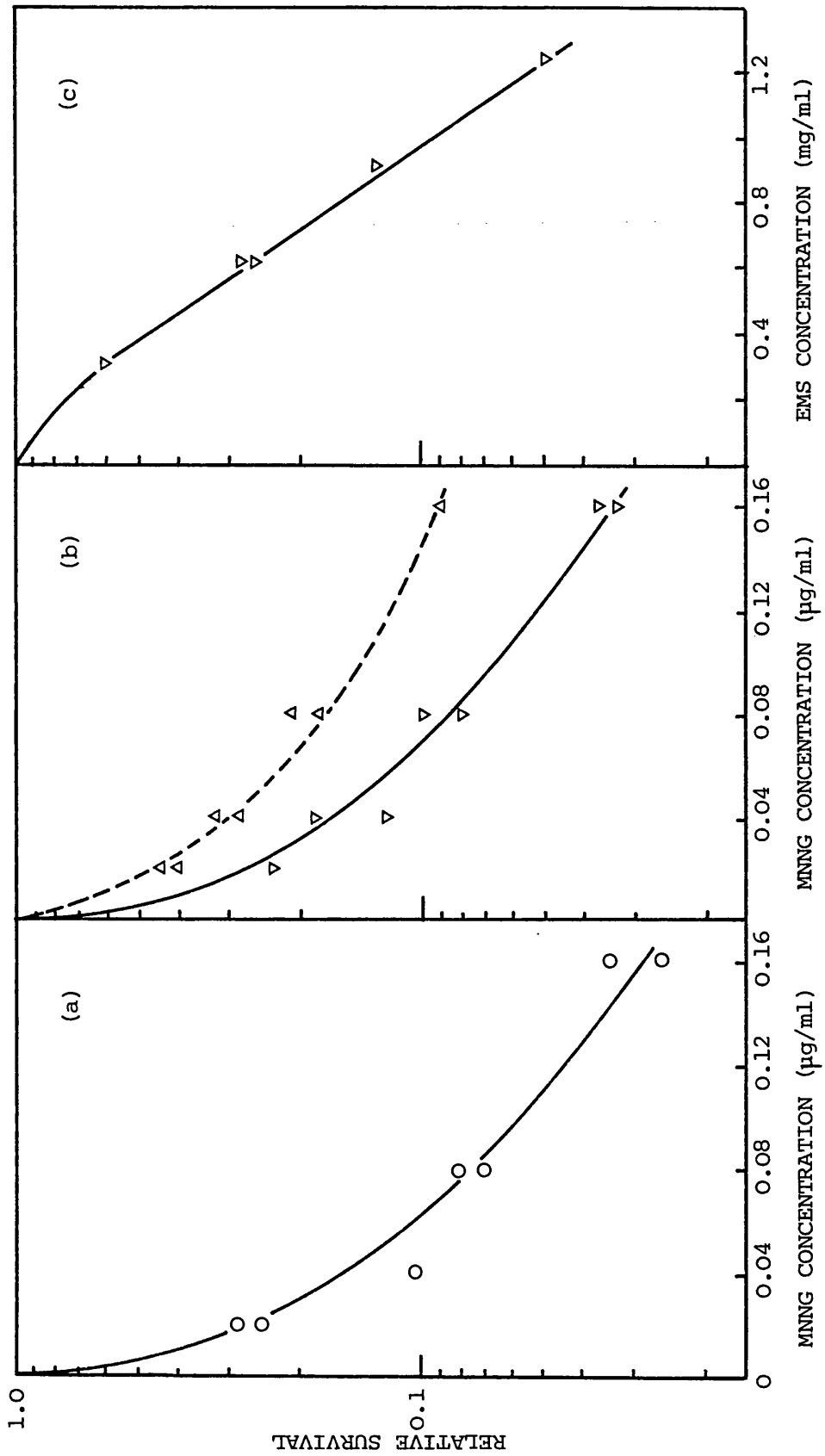


Fig. 5.1 Dose-response curves for single-plated V79-379A cells treated with (a) and (b) MNNG (c) EMS. Cells grown in F10 (O), EMEM (Δ), or EMEM+LC (∇).

for V79 cells in F10 (Fig. 5.1a), 0.011 μg/ml for cells in EMEM + LC and 0.03 μg/ml for cells in EMEM (Fig. 5.1b). The D_{37} value for EMS was 0.49 mg/ml (Fig. 5.1c).

5.2.2 The effect of TPA on spontaneous and MNNG-induced Oua^R mutation of V79-379A cells under different culture conditions.

Oua^R mutations in V79 cells can be assessed under exactly the same conditions as used previously for CHO-K1 cells. Thus, the density of cells seeded onto selection plates was 2×10^5 cells/90 mm dish/10 ml medium, the mutation expression time was 49 hr and the mutant selection time was 8 days. In these studies, however, the concentration of Oua in selection medium was 1 mM. Similar selection conditions have been used previously in Oua^R mutation assays with other V79 cells (Bradley et al., 1981).

The effects of TPA were examined at a single concentration of 1 μg/ml. This was added to plates 2 hr after MNNG treatment and maintained in culture medium for the remainder of the mutation expression and the whole of the mutant selection. The MNNG concentration used throughout was 0.04 μg/ml.

The effects of TPA were examined in V79-379A cells grown in three culture media - F10, EMEM and EMEM + LC. Prior to their use in experiments the cells were grown for at least two passage intervals in the appropriate culture medium. The effects of TPA on spontaneous Oua^R mutations was also assessed, thus four treatment groups were included in each experiment - control, TPA, MNNG and MNNG + TPA. The experimental procedure followed that described in 3.3.2 and illustrated in Fig. 3.4.

The data obtained for duplicate experiments for V79-379A cells grown in different culture media are given in Tables 5.1 - 5.3. In contrast to the reports of other workers, these results indicate that V79-379A cells may be sensitive to the cytotoxic effects of TPA. In all experiments TPA reduced the survival of both control and MNNG-treated cells. However, in agreement with previous studies in V79 TPA alone was not mutagenic in V79-379A cells. TPA did increase the frequency of Oua^R V79-379A mutants following MNNG treatment and this mutagenesis enhancing activity for TPA was independent of culture conditions.

5.2.3 The effect of TPA on spontaneous and EMS-induced Oua^R mutation of V79-379A cel ls.

The effects of TPA on EMS-induced Oua^R mutations was investigated in V79-379A cells grown in EMEM + LC. The concentration of EMS used was 0.8 mg/ml. The experimental procedure was that employed for MNNG studies (5.2.2), except that a terminating medium change was necessary following a 2 hr mutagen exposure.

The data obtained for this experiment are given in Table 5.4. These results show that TPA is not toxic to either control or EMS-treated V79-379A cells. TPA *per se* was not mutagenic but it did enhance the frequency of EMS-induced Oua^R mutations.

5.2.4 The effect of TPA on metabolic cooperation between Oua^R and Oua^S V79-379A cells

V79 cells have been reported to metabolically cooperate (Yotti et al., 1979), and an inhibition of this process may explain the mutagenesis enhancing activity of TPA in the V79-379A cell line. Thus, the effects of TPA on the recovery of a known number of Oua^R V79-379A

Table 5.1 The effect of TPA(1μg/ml) on the incidence of spontaneous and 0.04μg/ml MNNG-induced Oua^R mutation of V79-379A cells grown in F10.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control	62.3	1	1.6
TPA	45.8	1	2.2
MNNG	5.3	6	111
MNNG + TPA	5.6	11	195*
Cells/plate - 2.03×10^5			
Plates/treatment - 5			
Expt 2			
Control	60.6	1	1.6
TPA	53.5	0	0
MNNG	7.6	6	78
MNNG + TPA	4.8	12	248*
Cells/plate - 2.02×10^5			
Plates/treatment - 5			

* Significantly higher than MNNG alone. $p<0.05$

Table 5.2 The effect of TPA(1μg/ml) on the incidence of spontaneous and 0.04μg/ml MNNG-induced Oua^R mutation of V79-379A cells grown in EMEM.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control	80.8	0	0
TPA	49.3	2	4
MNNG	17.5	128	719
MNNG + TPA	5.6	89	1577*
Cells/plate - 2.03×10^5			
Plates/treatment - 5			
Expt 2			
Control	64.1	1	1.5
TPA	45.3	3	6.6
MNNG	24.5	76	307
MNNG + TPA	17.0	130	757*
Cells/plate - 2.02×10^5			
Plates/treatment - 5			

* Significantly higher than MNNG alone. $p<0.005$

Table 5.3 The effect of TPA(1μg/ml) on the incidence of spontaneous and 0.04μg/ml MNNG-induced Oua^R mutation of V79-379A cells grown in EMEM+LC.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control	63.0	1	1.6
TPA	55.1	1	1.8
MNNG	9.4	50	532
MNNG + TPA	4.9	97	1980*
Cells/plate - $2x10^5$			
Plates/treatment - 5			
Expt 2			
Control	73.0	0	0
TPA	54.4	3	5.5
MNNG	22.5	80	355
MNNG + TPA	15.3	114	744*
Cells/plate - $2x10^5$			
Plates/treatment - 5			

* Significantly higher than MNNG alone. $p<0.005$

Table 5.4 The effect of TPA(1μg/ml) on the incidence of spontaneous and 0.8mg/ml EMS-induced Oua^R mutation of V79-379A cells grown in EMEM+LC.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Control	62.5	2	3.1
TPA	63.3	2	3.1
EMS	12.9	96	726
EMS + TPA	13.3	181	1337*

Cells/plate - 2.05×10^5
Plates/treatment - 5

* Significantly higher than EMS alone. $p<0.005$

cells cocultured with wild-type (Oua^S) cells was examined in a reconstruction experiment.

(a) Isolation of Oua^R V79-379A strains:

For the proposed reconstruction experiment it was necessary to isolate and propagate strains of Oua^R V79-379A cells.

2×10^5 V79-379A cells at passage 5 were inoculated into two 90 mm T /C dishes with 10 ml EMEM + LC and incubated for 4 hr. MNNG (0.04 µg/ml) was then added to the culture medium. 49 hr after the addition of MNNG the medium in both plates was replaced with 10 ml 1 mM Oua EMEM + LC. Plates were incubated for a further 8 days, then one discrete Oua^R colony was isolated from each plate following the method given in 3.3.4a.

These two independent cell strains were designated V79-379A Oua 3 and V79-379A Oua 4.

(b) Reconstruction experiment:

The experimental protocol followed that described in 3.3.4b, except that V79-379A cells were grown in EMEM + LC and the concentration of Oua in selection medium was 1 mM. The Oua^S cells were provided by a 48 hr culture of V79-379A cells at passage 8. The Oua^R cells were provided by a 48 hr culture of V79-379A Oua 3 cells also at passage 8.

The numbers of cells plated, the percentage of Oua^R colonies recovered and the plating efficiencies of unselected controls are given in Table 5.5. This data indicates that in the absence of wild-type cells TPA was not toxic to Oua^R V79-379A cells.

In the presence of 2×10^5 wild-type cells the recovery of Oua^R colonies was decreased from 63.2% to 34.3%. However, when TPA was also present the percentage of Oua^R colonies recovered was similar to that

Table 5.5 The effect of TPA(1µg/ml) on the recovery of Oua^R V79-379A cells in the presence of wild-type Oua^S cells grown in EMEM+LC.

WILD-TYPE CELLS(a) ($2x10^5$)	OUA^R CELLS(b) (100)	TPA (1µg/ml)	% RECOVERY(c) OF OUA^R
-	+	-	63.2
-	+	+	63.0
+	+	-	34.3
+	+	+	56.2

(a) Plating efficiency Oua^S cells - 62.4%

(b) Plating efficiency Oua^R cells - 64.1%

(c) Plates/treatment - 5

in the absence of wild-type cells. An inhibition of metabolic cooperation may thus provide an explanation for the enhancement of Oua^R mutations by TPA in V79-379A cells.

5.3 The effect of TPA on MNNG-induced mutation of CHO cells to Oua^R

5.3.1 Toxic response test: toxicity of MNNG to single-plated CHO(S) cells.

As an *in situ* protocol would again be employed for the assessment of Oua^R mutation, the toxicity of MNNG was determined in single-plated CHO(S) cells grown in F10. Initially MNNG concentrations were selected from the survival data reported by Kao and Puck (1968) and Bempong (1979) for other CHO cells. In preliminary studies, however, these doses were found to be too toxic towards CHO(S) cells and the doses were reduced by a factor of 10. The MNNG concentrations selected for this study were in the range 0.01 - 0.08 µg/ml.

The experimental procedure followed that used previously for toxicity studies in CHO-K1A cells (see 3.3.1), except that MNNG treatment was assumed to be self-limiting and a terminating medium change unnecessary.

The data obtained are represented in a dose-response curve in Fig. 5.2. In contrast to the curves obtained for CHO-K1A (Fig. 3.1a) and V79-379A (Fig. 5.1a), the dose-response curve for CHO(S) cells treated with MNNG shows an exponential reduction with no change in gradient at low survival levels. By interpolation, the D_{37} value for MNNG is 0.011 µg/ml.

5.3.2 The effect of TPA on spontaneous and MNNG-induced Oua^R mutation of CHO(S) cells.

As for studies with CHO-K1A and V79-379A cells, Oua^R mutations in CHO(S) cells were assessed using the *in situ* protocol described in 3.3.2, except that the concentration of Oua in selection medium was 1 mM. Similar selection conditions have been used in Oua^R mutation assays with other CHO cells (Hsie et al., 1981). The concentration

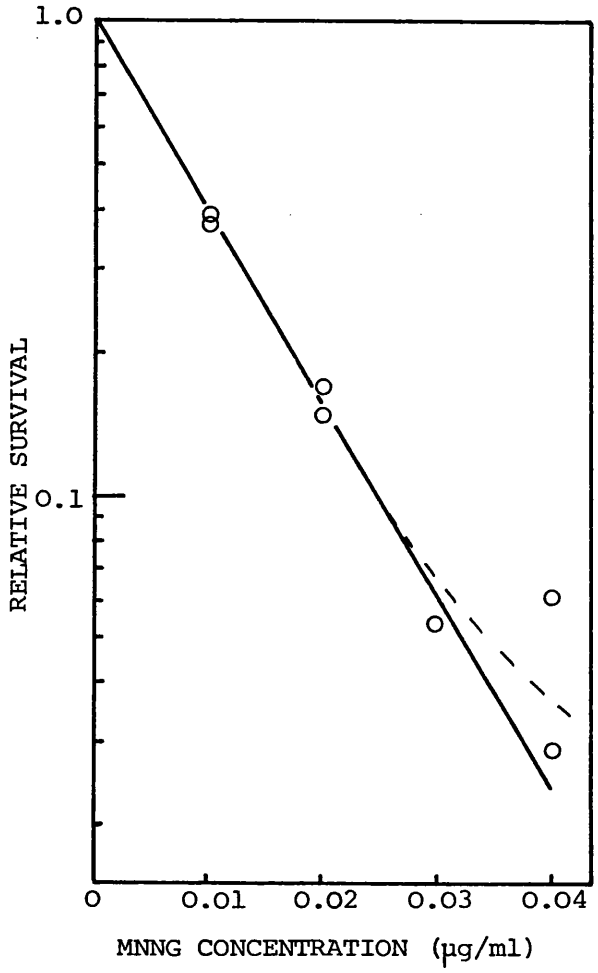


Fig. 5.2 Dose-response curve for single-plated CHO(S) cells grown in F10 treated with MNNG.

of MNNG used was 0.02 μg/ml. TPA at a concentration of 1 μg/ml was added 2 hr after MNNG treatment, and maintained in the culture medium for the remainder of mutation expression and the whole of mutant selection.

The effects of TPA on spontaneous Oua^R mutations was also assessed in CHO(S) cells, therefore, four treatment groups were included - control, TPA, MNNG and MNNG + TPA.

The data obtained for duplicate experiments, for CHO(S) cells grown in F10, are given in Table 5.6. In CHO(S) cells TPA alone was neither toxic nor mutagenic. Furthermore, it did not affect the lethal effects of MNNG. In contrast to its action in other cell lines, TPA did not enhance the expression of MNNG-induced Oua^R mutations in the CHO(S) cell line. This is in agreement with the results presented by Thompson et al. (1980) that TPA does not enhance mutagenesis in CHO cells.

Table 5.6 The effect of TPA(1μg/ml) on the incidence of spontaneous and 0.02μg/ml MNNG-induced Oua^R mutation of CHO(S) cells grown in F10.

TREATMENT	% PLATING EFFICIENCY	TOTAL MUTANTS	MUTANTS/10^6 SURVIVORS
Expt 1			
Control	102.3	3	2.9
TPA	91.0	1	1.1
MNNG	13.9	10	72
MNNG + TPA	14.6	9	62
Cells/plate - $2x10^5$			
Plates/treatment - 5			
Expt 2			
Control	93.8	2	2.1
TPA	85.1	3	3.5
MNNG	17.2	19	110
MNNG + TPA	24.8	21	84
Cells/plate - $2.02x10^5$			
Plates/treatment- 5			

5.4 Conclusions

From the results presented in this chapter the following can be concluded.

1. Both V79-379A and CHO(S) cells were considerably more sensitive to the lethal effects of MNNG than other V79 and CHO cells.
2. In agreement with previous reports, TPA was neither toxic nor mutagenic in V79-379A or CHO(S) cells.
3. The mutagenesis enhancing effect of TPA is cell line specific. TPA enhanced MNNG-induced mutation of V79-379A cells to Oua^R (5.2) but not MNNG-induced Oua^R mutations in CHO(S) cells (5.3).*
4. The mutagenesis enhancing activity of TPA in V79-379A cells was neither mutagen specific nor dependent on culture conditions.
5. An inhibition of metabolic cooperation between Oua^R and Oua^S cells may explain the mutagenesis enhancing activity of TPA in V79-379A cells.

These conclusions will be discussed, together with those from the other experimental chapters, in Chapter 7.

* These conclusions are based on the assumption that the treatment conditions employed are optimal for both V79-379A and CHO(S) cells.

CHAPTER 6. THE EFFECTS OF TPA ON THE INACTIVATION OF UV IRRADIATED CHO-K1 CELLS

6.1 Introduction

The ultraviolet (UV) spectrum is conventionally divided into three regions which have distinct physical and biological properties.

Near-UV (UVA)	400 nm - 320 nm
Mid-UV (UVB)	320 nm - 290 nm
Far-UV (UVC)	less than 290 nm.

These regions are usually represented by 'typical' wavelengths of UV radiation based on the emission lines of mercury vapour lamps; UVA 365 nm, UVB 313 nm and UVC 254 nm. Studies in bacterial and mammalian cells in culture indicate that the spectrum of lesions induced by UV changes as wavelength increases (Jagger,1985). Thus, the biological effects of near-UV radiation are now recognised to be distinct from those of far-UV radiation.

In the far-UV region, there is good evidence that the radiation is directly absorbed by DNA and this causes the formation of photo-products such as the pyrimidine dimer (Varghese and Wang, 1967; Brash and Heseltine, 1982). The action spectra for a number of biological end points in rodent, frog and human cells have been correlated with the induction of these dimers (reviewed by Jagger, 1985). Thus, pyrimidine dimers are now accepted as the lesions responsible for the lethal and mutagenic effects of far-UV.

With increasing wavelength it is apparent that other forms of damage to both DNA and other cellular components may become important. Although evidence indicates that DNA is still the primary site of lethal damage (Webb, 1977; Jagger, 1981; 1985), there are suggestions that membrane damage may be important for near-UV-induced cell inactivation (Moss and

Smith, 1981; Ito and Ito, 1983; Kelland et al., 1983a, 1983b,1984).

Mid-UV is regarded as a transition region and its action seems to be a mixture of far- and near-UV effects (Jagger, 1985).

On the basis of a possible membrane action for UV radiation it was proposed to investigate the membrane activity of TPA in CHO-K1 cells. The rationale for these investigations was that cells with damaged membranes are likely to be more susceptible to other forms of membrane-mediated toxicity. This has previously been used as the basis for studies into the membrane effects of UV radiation in bacteria (Moss and Smith, 1981; Kelland et al., 1983a, 1983b). The effects of TPA on the inactivation of CHO-K1A cells following irradiation with UV light of 3 different wavelengths, representing the distinct spectral regions, was examined.

6.2 Equipment for UV irradiation experiments

6.2.1 Dark room

All experiments employing UV light were carried out in a 'dark room' under illumination from red fluorescent tubes (Atlas Ltd., 80W) emitting only light of wavelengths greater than 500 nm.

6.2.2 Radiation sources

i) Penray lamp (Ultraviolet Products Inc.) - a 5 cm low pressure mercury lamp fitted with an integral filter (G-275). This is designed to isolate the 253.7 nm line of the mercury spectrum and about 92-97% of lamp output is at this wavelength. This lamp provides a 254 nm dose rate of 0.073 J $m^{-2}s^{-1}$.

ii) Bausch and Lamb SP 200 lamp (Bausch and Lamb, Rochester, New York) - a 200 W high pressure mercury vapour lamp with a fused silica envelope and an appropriate fused silica condensing lens system. Lamps were replaced after 100 hr use and a 2 hr burn off was allowed for newly fitted lamps prior to their use for irradiation. This light source was used in conjunction with a Bausch and Lamb high intensity grating monochromator, and can be operated at a wavelength range from 200 nm - 800 nm.

6.2.3 Arrangement of optical bench

The general arrangement of the apparatus for irradiation of cell suspensions is given in Fig. 6.1(a). This refers specifically to the Bausch and Lamb SP 200 source, although only minor differences in detail occurred when the penray source was employed. All optical components were arranged in a horizontal plane on an Ealing Beck optical bench and associated supports, allowing vertical and horizontal adjustments.

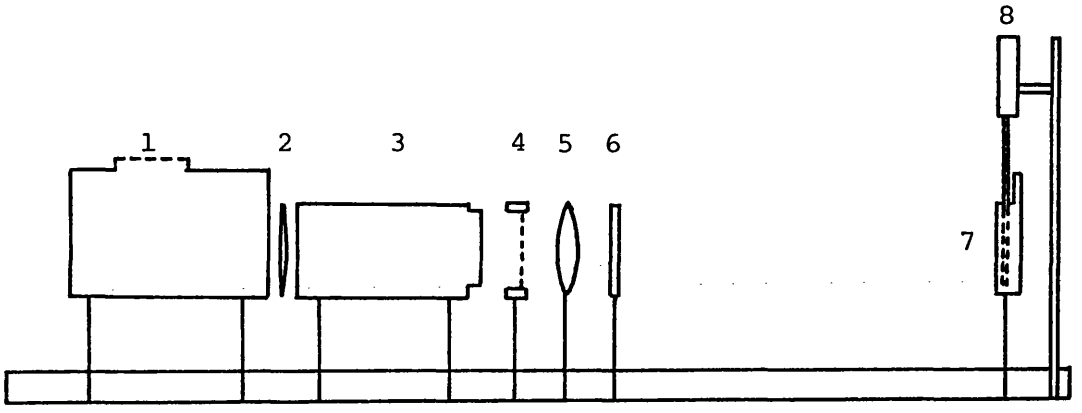


Fig. 6.1a Diagram of the optical bench for UV irradiation of cells in suspension.

1 Mercury UV source (SP200)
2 Quartz collective lens
3 Monochromator
4 Shutter
5 Focusing lens
6 Stray light filter
7 Irradiation cuvette (see below)
8 Stirrer

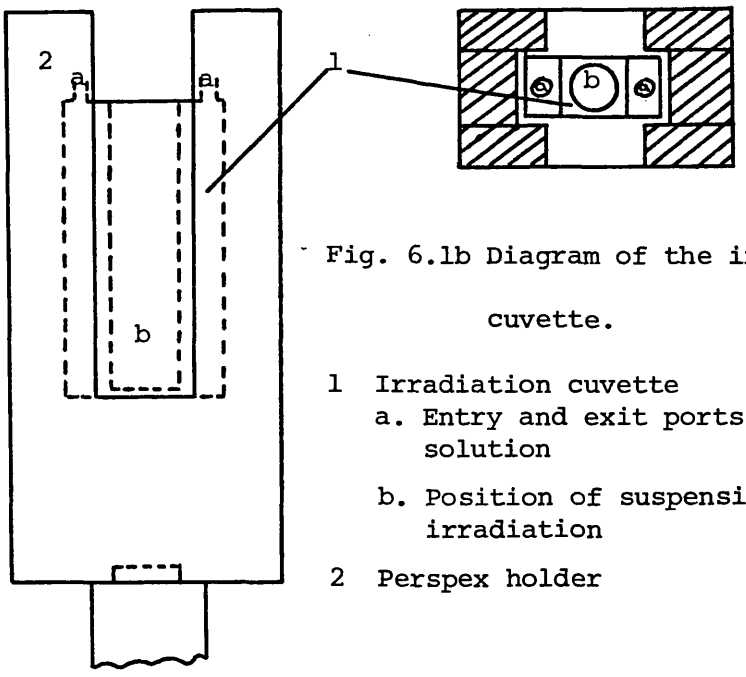


Fig. 6.1b Diagram of the irradiation cuvette.

1 Irradiation cuvette
a. Entry and exit ports for cooling solution
b. Position of suspension for irradiation
2 Perspex holder

i) The shutter was an iris camera shutter (G.B. Kershaw 630) with a 2 cm aperture and was fitted with a cable release.

ii) The focusing lens used only in conjunction with the Bausch Lamb SP 200 source. This was a 40 mm diameter Spectrosil biconvex lens having a focal length of 55 cm. This produced an inverted magnified image of the exit slit of the monochromator 3 cm long and 1 cm wide allowing full illumination of the irradiation cuvette.

iii) Stray light filters. During irradiation at wavelengths longer than 254 nm using a monochromator it is essential that any stray light of shorter wavelengths is removed from the irradiation beam. This prevents errors in the biological sensitivity measured at a particular wavelength due to inactivation by shorter wavelength irradiations. An appropriate UV absorbing filter was thus positioned between the monchromator and the irradiation cuvette. Details of the stray light filters used for each wavelength are given in Table 6.1.

Table 6.1 Stray light filters used in irradiation experiments

WAVELENGTH (nm)	FILTER CUT-OFF ON LOW SIDE (nm)	STRAY LIGHT FILTER
254	-	G-275
313	301	Mylar C (2.5μm)
365	332	Corning 0-52

(iv) Irradiation cuvette - a jacketed 10 mm internal width, 10 mm path length quartz cuvette was used (Thermal Syndicate Ltd.), illustrated in Fig. 6.1(b). The temperature of the cell suspension was controlled by the circulation of 25% ethylene glycol solution through the cuvette jacket. For irradiations at 0°C an insulated 20 litre

bath of glycol solution was cooled by a U-cool refrigeration unit (Neslab Instruments Ltd.). This solution was circulated at a high flow rate through lagged rubber tubing by a peristaltic pump (Watson Manlow Ltd.). The cuvette temperature was monitored by a probe thermometer (Comark Ltd.) and a temperature of 0°C ± 0.5°C was always maintained.

Condensation which formed on the cuvette faces during irradiation was removed at the sampling interval with a clean tissue.

(v) The stirrer. Cell suspensions were stirred during irradiation by means of a quartz paddle in a laboratory stirrer (Stanhope Seta Ltd.) at approximately 600 rpm.

(vi) Fluence rate determinations. The fluence rates of UV radiation received by cell suspensions were measured using a calibrated Oriel 7102 thermophile.

For use on the optical bench the thermopile was mounted on a sliding bench saddle beside the cuvette such that the detector was coincident with the inside front face of the cuvette when moved across into the radiation beam. Output voltages were measured on a micro-voltameter (Kiethley Instruments Ltd.).

6.3 The inactivation of CHO-K1 cells by far-, mid- and near-UV radiations

Although the UV inactivation of CHO and CHO-K1 cells to monolayer has been reported (Zelle et al., 1980; Dewdney, 1982) no data are available for the response of CHO-K1 cells irradiated in suspension. The inactivation of CHO-K1A cells by the three wavelengths to be used (254 nm, 313 nm and 365 nm) was investigated prior to an examination of the effects of TPA on cell survival. The dose ranges for each wavelength were suggested by studies for V79 cells irradiated under similar conditions (Rothman and Setlow, 1979; Wells and Han, 1985).

6.3.1 Preparation of cell suspensions for irradiation experiments.

As irradiation of culture medium in the near-UV region leads to the formation of toxic photoproducts (Wang et al., 1974), cell suspensions were prepared in PBS.

5×10^5 CHO-K1A cells were inoculated into a 150 ml culture bottle with 15 ml F10 and incubated. After 48 hr growth, the cell monolayer was dispersed with trypsin (2.8.2) and the cells transferred with 5 ml PBS to a 16 mm T/C tube. Cells were sedimented by centrifugation (1500 rpm; 5 min) and resuspended in 3 ml fresh PBS. Cell density was determined by haemocytometer count and adjusted to 2×10^5 cells/ml with further PBS.

6.3.2 Procedure for UV irradiation of cells in suspension.

Before use time was always allowed for the radiation source to 'warm up' and for the UV output to stabilise, in the case of the penray lamp 20 min and for the Bausch and Lamb lamp at least 30 min. The irradiation cuvette was then positioned on the optical bench at a height

and distance from the source such that its front face was within the field of illumination. The thermopile was then moved into the radiation beam and a number of microvolt readings were taken by using the shutter. The mean of these readings was used to calculate the incident fluence rate from the formula:

$$\text{Fluence rate} = \text{thermopile calibration factor} \times \text{mean microvolt reading}$$

The calibration factor for the Oriel 7102 thermopile was 0.0267 $Wm^{-2}\ mV^{-1}$.

The irradiation cuvette was moved back into position and rinsed twice with sterile PBS. The stirring paddle was then moved into position and switched on. The cuvette was sterilised, with the stirrer in motion, by a 15 min exposure to the 254 nm output of a penray lamp.

A 3 ml volume of cell suspension (see 6.3.1) was then transferred to the sterile cuvette and the circulation of the cooling liquid through the jacket started. 10 min was allowed for the cell suspension to reach the desired temperature (0°C). The cell suspension was then exposed to graded fluences of UV radiation.

For the assessment of viability a 0.2 ml sample of cell suspension was removed from the cuvette both as a control and after each fluence. Control and irradiated cell samples were diluted in complete medium, F10. The dilution employed depended upon the expected sensitivity of the cells to UV radiation. 0.1 ml of these dilutions were plated into triplicate 50 mm T/C dishes containing 4.9 ml F10, so that 20 to 200 macroscopic colonies were visible after incubation. Where the sensitivity of the cells was uncertain cells were plated at more than one density. Seeded dishes were placed into incubation boxes, gassed, sealed and incubated in the dark. After 7 days the colonies were stained and scored in the usual way (2.8.8).

The inactivation curves for CHO-K1A cells following irradiation with 254 nm, 313 nm and 365 nm UV using the procedure outlined above are given in Fig. 6.2. The survival curves for CHO-K1A cells at the wavelengths examined are of the shouldered type. These are similar to those of other cell lines irradiated in suspension (Wells and Han, 1985). The D_{37} values can be interpolated from these curves. They are 44 Jm^{-2} at 254 nm (Fig. 6.2a), 20 kJm^{-2} at 313 nm (Fig. 6.2b) and 220 kJm^{-2} at 365 nm (Fig. 6.2c).

Compared to CHO-K1A cells irradiated as monolayers (D_{37} value 36.5 Jm^{-2}, Dewdney, 1982), CHO-K1A cells in suspension show a similar response to the lethal effects of 254 nm UV. At both 254 nm and 313 nm CHO-K1A cells are more resistant to UV inactivation than V79 cells. The D_{37} values reported for V79 cells under similar conditions at these wavelengths are 15 Jm^{-2} and 12 kJm^{-2}, respectively (Wells and Han, 1985). At 365 nm, however, CHO-K1A cells are slightly more sensitive than V79 cells (D_{37} 300 kJm^{-2}, Han et al., 1984).

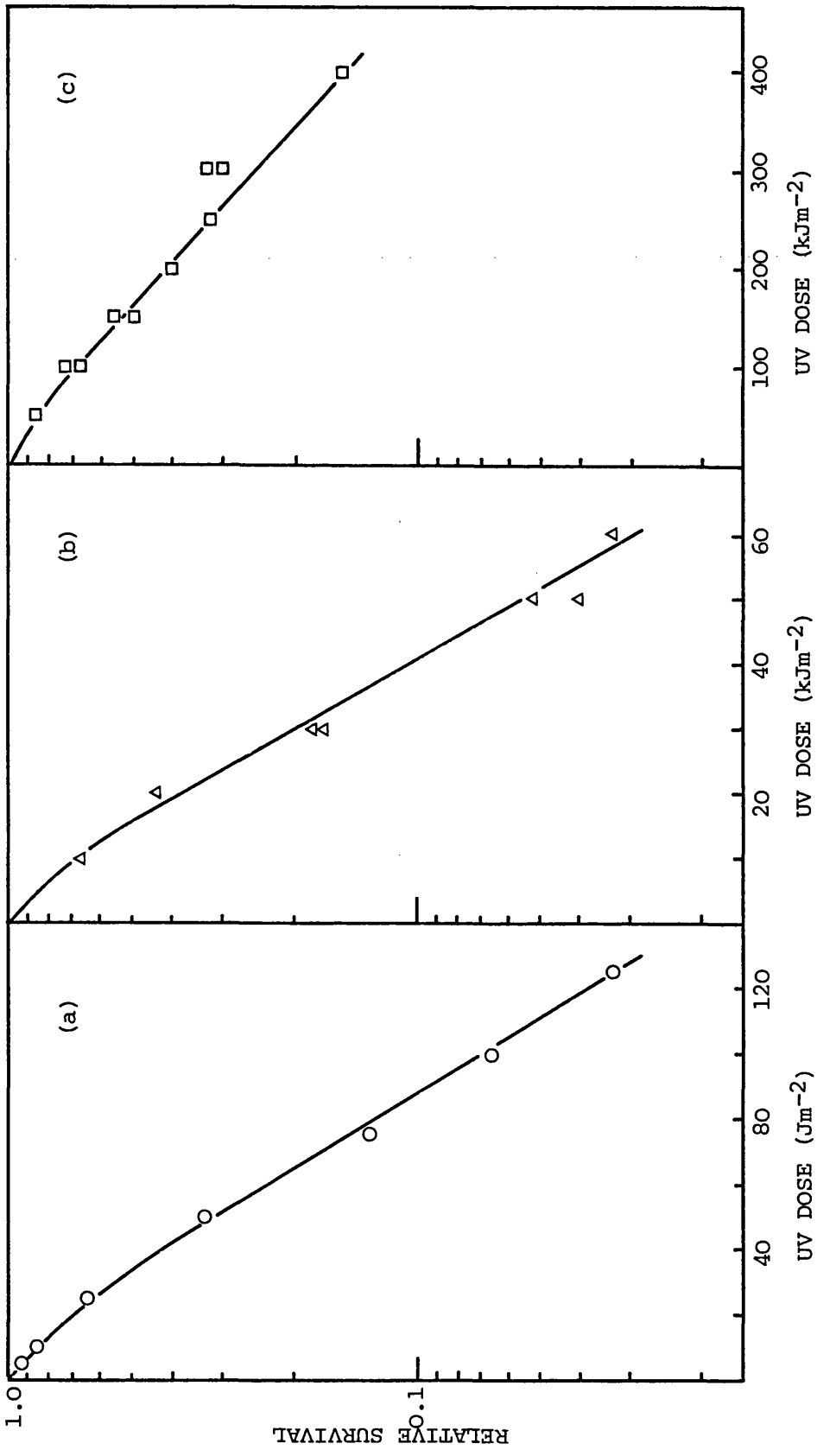


Fig. 6.2 Inactivation curves for CHO-K1A cells irradiated in suspension at 0°C with (a) 254nm (b) 313nm (c) 365nm ultraviolet light.

6.4 The effects of TPA on the inactivation of CHO-K1 cells by far-, mid- and near-UV radiations

To assess its effect on the survival of CHO-K1A cells following UV irradiation TPA was added to the plating medium at a concentration of 1 μg/ml. This concentration has been repeatedly shown as non-toxic to normal CHO-K1A cells (see Chapters 3 and 4).

The experimental procedure used was that given above (6.3.2), except following dilution 0.1 ml aliquots of the appropriate diluted suspension were inoculated into six 50 mm T/C dishes containing 4.9 ml F10. TPA or TPA vehicle was then added to three dishes for each fluence sample. Seeded dishes were boxed, gassed, sealed and incubated in the dark for 7 days. Colonies were stained and scored in the usual way. The effects of TPA on the inactivation of CHO-K1A cells following radiation with 254 nm, 313 nm and 365 nm UV are given in Figs. 6.3, 6.4 and 6.5, respectively. The results in Figs. 6.3 and 6.5 represent a single experiment for each wavelength, while those in Fig. 6.4 represent duplicate experiments at 313 nm. The control plating efficiencies for untreated and TPA treated cells, also included in these figures, show that TPA was not toxic to CHO-K1A cells in the control groups.

The cells irradiated with either 313 nm (Fig. 6.4) or 365 nm (Fig. 6.5) UV are clearly sensitized to the radiation treatment in the presence of TPA. However, the presence of TPA in the plating medium did not affect the response of CHO-K1A cells to 254 nm UV radiation (Fig. 6.3).

All of the inactivation curves are of the shouldered-type obtained in 6.3. Their exponential regions can be described by the equation

$$N_D = N_o e^{-kD}$$

where N_D is the surviving fraction after dose D, N_o the surviving fraction after dose 0, and k the inactivation factor. Quantification

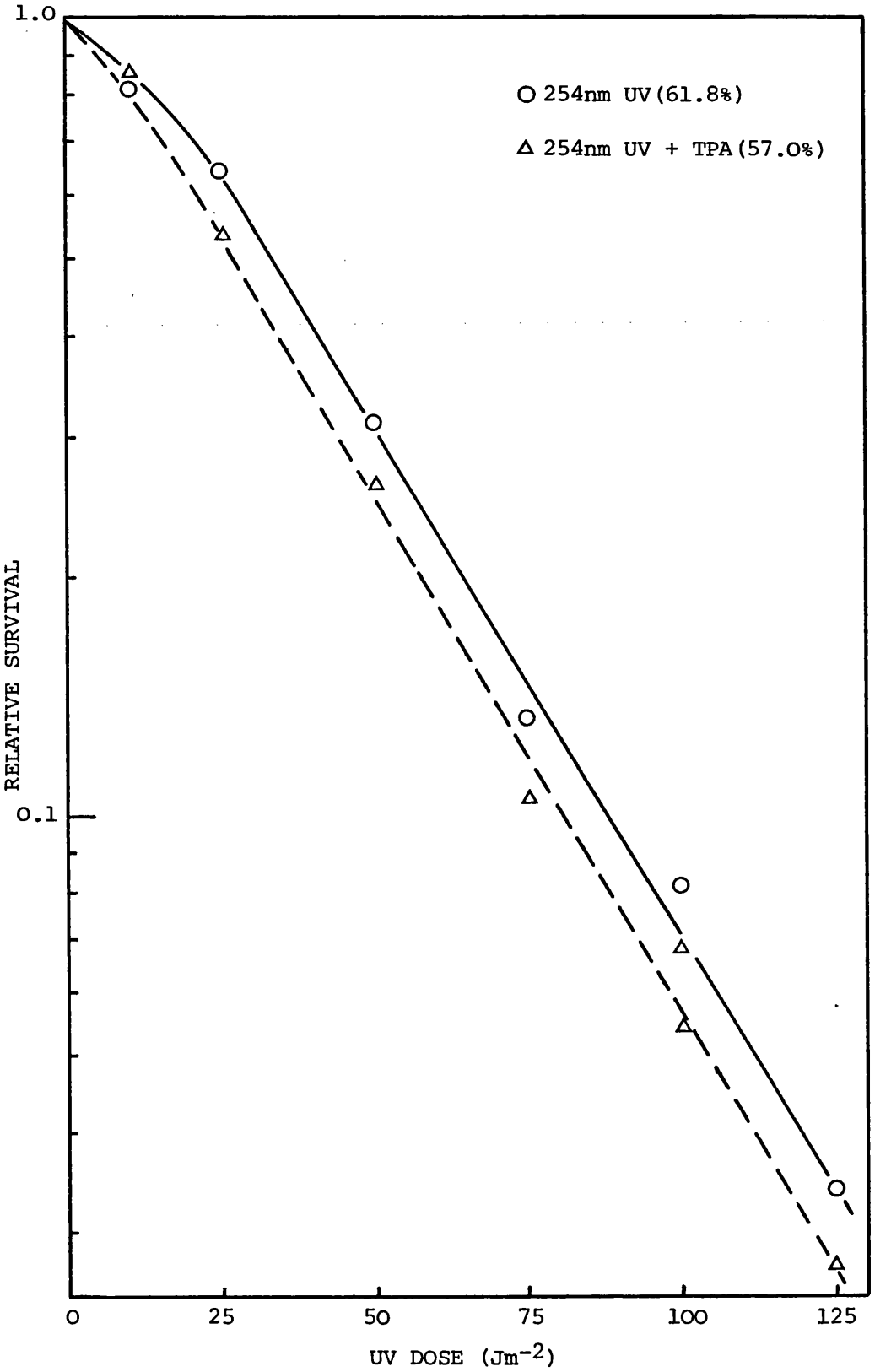


Fig. 6.3 The effect of TPA(1μg/ml) in the plating medium on the survival of CHO-K1A cells irradiated in suspension at 0°C with 254nm ultraviolet light.

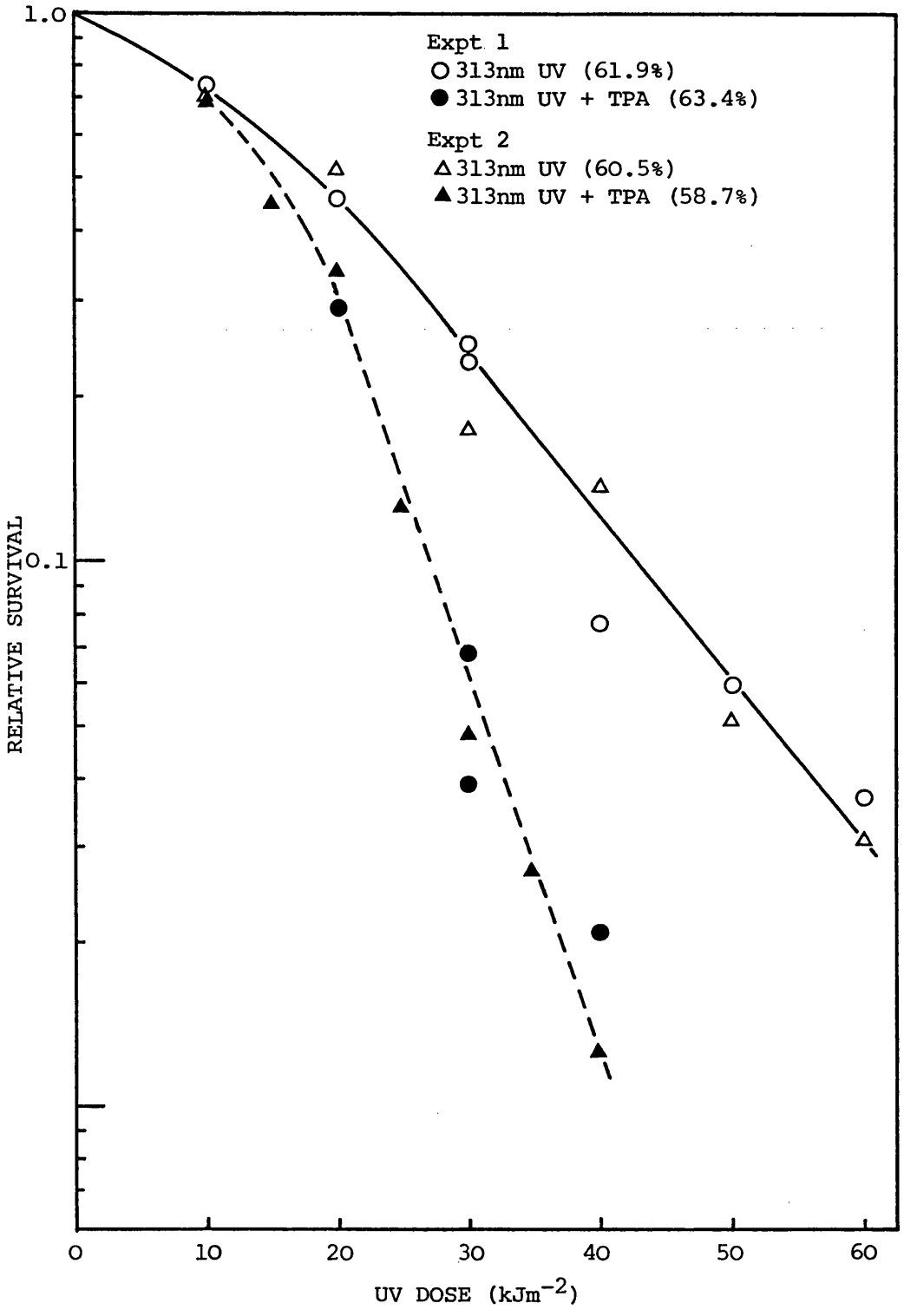


Fig. 6.4 The effect of TPA(1μg/ml) in the plating medium on the survival of CHO-K1A cells irradiated in suspension at 0°C with 313nm ultraviolet light.

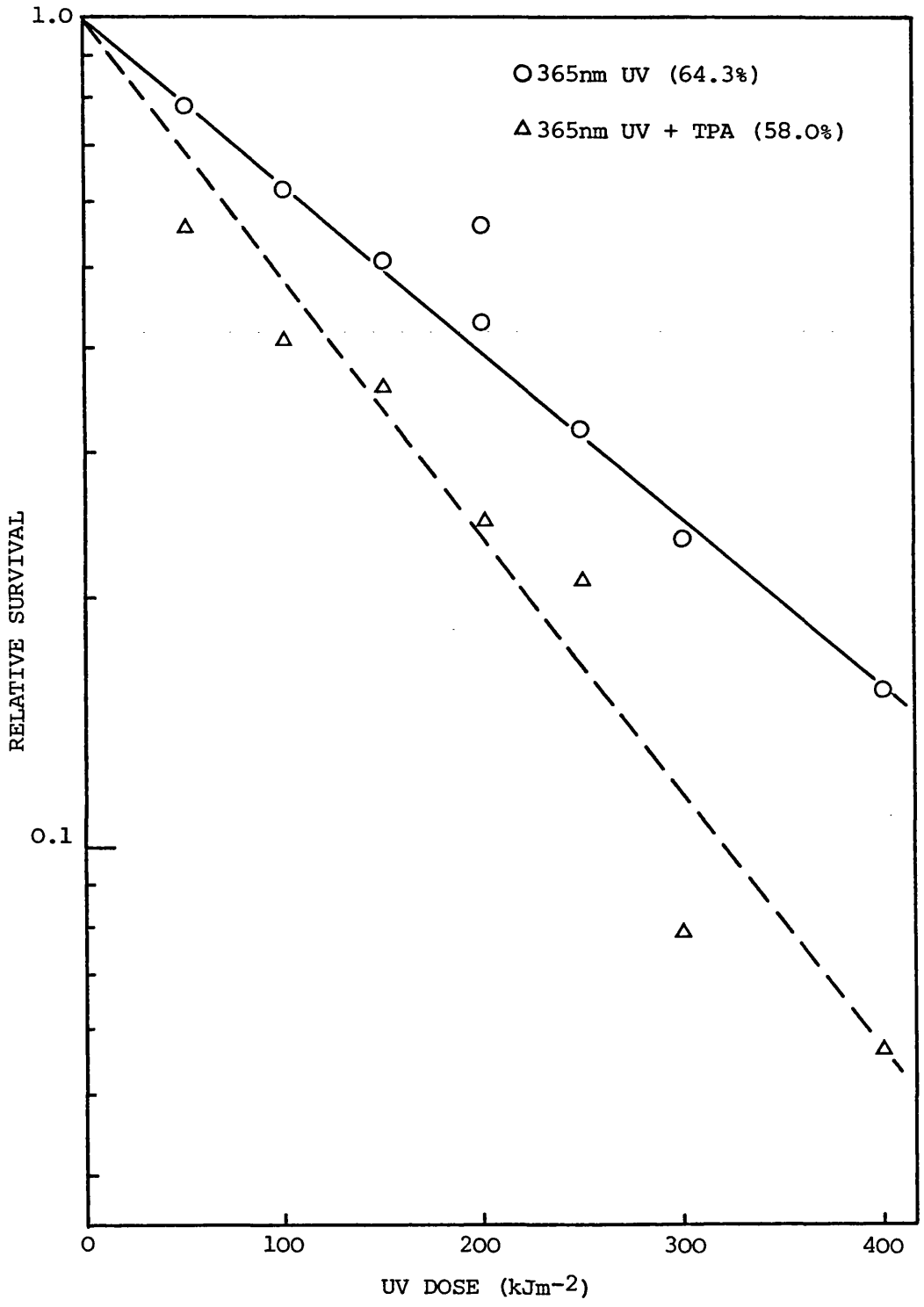


Fig. 6.5 The effect of TPA(1μg/ml) in the plating medium on the survival of CHO-K1A cells irradiated in suspension at 0°C with 365nm ultraviolet light.

of the effects of TPA at the different wavelengths is thus possible by comparing the k values of the curves. The inactivation factors calculated from Figs. 6.3 - 6.5 are given in Table 6.2, together with the ratio of TPA treated to untreated inactivation factors for each wavelength. These results indicate that TPA has a greater effect on the inactivation of CHO-K1A cells following irradiation with 313 nm than with 365 nm UV.

Table 6.2 Quantification of the effects of TPA(1μg/ml) on the survival of CHO-K1A cells following irradiation in suspension at 0°C with UV.

UV WAVELENGTH (nm)	TPA	k VALUE ($^1/Jm^{-2}$)	RATIO $\frac{k_{TPA}}{k_{Control}}$
254 (Fig. 6.3)	-	-4.23×10^{-2}	1.01
	+	-4.29×10^{-2}	
313 (Fig. 6.4)	-	-9.76×10^{-5}	2.38
	+	-2.32×10^{-4}	
365 (Fig. 6.5)	-	-1.05×10^{-5}	1.96
	+	-2.06×10^{-5}	

6.5 The effect of TPA on the survival of CHO-K1 cells after prolonged holding in PBS

For both 313 nm and 365 nm prolonged irradiation times of approximately 210 min are required for the induction of their lethal effects in CHO-K1A cells. It is possible that the effects of TPA observed above may be due to its action on cells weakened by prolonged holding in PBS. The effects of TPA in the plating medium on the survival of unirradiated CHO-K1A cells following a prolonged holding in PBS was examined.

A suspension of 2×10^5 CHO-K1A cells/ml was prepared in PBS (6.3.1) and held in the sterile irradiation cuvette at 0°C for 210 min. The suspension was stirred throughout at 600 rpm. For the assessment of viability 0.2 ml samples were removed from the cuvette at 30 min intervals. Cells were diluted and inoculated into F10 medium containing TPA or TPA vehicle as before. Plates were incubated in the dark, then stained and scored in the usual way after 7 days.

The results of this experiment are given in Table 6.3.

These results indicate that there is no reduction in the survival of CHO-K1A cells held in PBS, whether TPA is present in or absent from the plating medium. The results obtained in 6.4, therefore, represent a true interaction between UV irradiated cells and TPA, rather than experimental artefacts.

Table 6.3 The effect of TPA(1μg/ml) on cell viability following the holding of CHO-K1A cells in stirred PBS at 0°C.

HOLDING TIME (min)	SURVIVING FRACTION F10	F10 + TPA
0	1.00	1.00
30	1.04	0.96
60	1.01	0.97
90	1.07	0.95
120	0.97	0.96
150	0.96	1.01
180	0.96	0.97
210	0.96	0.95
Control plating efficiency	61.6%	63.0%

6.6 Conclusions

The following conclusions can be drawn from the work reported in this chapter.

1. The inactivation curves for CHO-K1A cells (Fig. 6.2) exhibit similar kinetics to those of other mammalian cell lines irradiated in suspension.
2. TPA does enhance UV inactivation of CHO-K1A cells, but only that caused by 313 nm (Fig. 6.4) and 365 nm (Fig. 6.5) UV radiation. In contrast to previous reports (Dewdney and Soper, 1984), TPA did not sensitize CHO-K1A cells to the cytotoxic effects of 254 nm UV (Fig. 6.3).
3. TPA sensitizes CHO-K1A cells to the cytotoxic effects of 313 nm UV to a greater extent than those of 365 nm UV (Table 6.2).
4. The effects of TPA cannot be attributed to an experimental artefact caused by the prolonged holding of CHO-K1A cells in PBS during irradiation (6.5).

These conclusions will be discussed, together with those from preceding experimental chapters, in Chapter 7.

CHAPTER 7. DISCUSSION

The initiation of two-stage carcinogenesis is generally accepted to be a mutational event.Thus, tumour promoters may act *in vivo* by enhancing the mutagenic consequences of carcinogen exposure. In support of this as a possible mechanism for tumour promotion it has been reported that the potent mouse skin tumour promoter TPA has such a mutagenesis enhancing activity in mammalian cells in culture. In V79 cells TPA enhanced gene mutations induced by UV or chemical agents at the Oua^R and TG^R loci (Lankas et al., 1977, 1980; Trosko et al., 1977). Likewise, this agent enhanced chemical-induced mutation to Oua^R in CHO-K1 cells (Dewdney and Soper, 1984). However, in CHO-K1 cells the activity of TPA was mutagen dependent, being observed with MNNG-induced mutation but not with mutations induced by EMS or UV. In both V79 and CHO-K1 cell lines TPA had no effect on spontaneous mutation frequencies or on mutagen-induced lethality. The aims of this present study were to further investigate this mutagen specific activity of TPA and to determine the mechanism of action of TPA as a mutagenesis enhancing agent in CHO-K1 cells.

To investigate the mutagen specificity it was envisaged that by using mutagens with different modes of action the type of mutagenic event potentiated by TPA could be inferred. Prior to their use in mutagenesis experiments the selected mutagens (1.7) were assessed for cytotoxic effects towards single-plated CHO-K1A cells (3.3.1). MNNG toxicity towards monolayer cultures of CHO-K1A cells was also determined (3.4.1).

The dose-response data for MNNG treatment of CHO-K1A cells (Figs. 3.1a and 3.5) are closely comparable with that obtained previously

for this cell line (Dewdney, 1982). When compared to parental CHO cells (D_{37} 0.44 μg/ml, Kao and Puck, 1968), single-plated CHO-K1A cells, and presumably other CHO-K1 cells, are more sensitive to the toxic effects of MNNG (D_{37} 0.021 μg/ml, Fig. 3.1a). This sensitivity may be a general response of CHO-K1 cells to N-nitroso agents. Single-plated CHO-K1A cells were also more sensitive to MNU (D_{37} 6.25 μg/ml, Fig. 3.1b) than CHO cells (D_{37} 48 μg/ml, Kao and Puck, 1968).

The dose-response curves for single-plated CHO-K1A cells treated with either N-nitroso agent (Figs. 3.1a and 3.1b) show an immediate simple exponential reduction in cell survival, followed by a marked decrease in gradient below 0.15 relative survival. Similar survival kinetics have been reported previously for CHO-K1A cells treated with MNNG (Dewdney, 1982). This may be due to the rapid decomposition of MNNG and MNU, as their reported half-lives in cell culture medium at 37°C are 14 and 8 min, respectively (Jensen et al., 1977). Alternatively, it has been suggested that a subpopulation resides within CHO-K1 cells which retains the resistant characteristics of parental CHO cells to these agents (Dewdney, 1982).

The toxicity of MNNG towards monolayer cultures of CHO-K1A cells is similar to that observed for another CHO-K1 subclone. The dose- response curve presented in Fig. 3.5 is superimposable upon that for CHO-K1-BH_4 cells reported by O'Neill et al. (1977).

The dose-response curves for single-plated CHO-K1A cells treated with MMS (Fig. 3.2a) and EMS (Fig. 3.2b) are of a shouldered-type and show similar kinetics to those reported by other workers for CHO-K1 cells (O'Neill et al., 1979; Dewdney, 1982). No valid comparison of D_{37} values can be made as the conditions employed in these present studies differ greatly, with respect to mutagen dose and exposure time, from those of other workers.

The presence of shoulders on the dose-response curves for these agents may be explained by one or more of the following.

1) The need for a minimum concentration of the agent in culture medium in order to overcome processes such as active transport, which prevent the agent reaching critical targets within the cell;

2) multiple structures within the cells which must be individually inactivated by the agent; or

3) cellular repair mechanisms whose capacity must first be saturated before damage can be elicited.

The toxic effects of the dialkylaminoalkyl chlorides (Fig. 3.3a) have not previously been reported in CHO-K1 cells. However, a similar rank order of toxicity, CEP > DMAE > DMAP, has been reported for these compounds in L5178Y mouse lymphoma cells (Thompson et al., 1981). When compared to the other mutagens assessed for toxicity during these present studies the dialkylaminoalkyl chlorides are relatively non-toxic to single-plated CHO-K1A cells.

The toxicity of DMBA was assessed in CHO-K1A cells without the inclusion of an exogenous activating system. The dose-response curve obtained (Fig. 3.3b), however, suggests that CHO-K1 cells may retain some endogenous metabolic activation activity for DMBA. A similar metabolic capability has been reported in parental CHO cells (Gupta and Singh, 1982). Using a 16 hr exposure these workers have shown DMBA concentrations of 25 μg/ml and 50 μg/ml to yield 84% and 76.4% relative survival, respectively, in CHO cells. Thus, CHO-K1A cells are markedly more sensitive to the toxic effects of this mutagen, as a 2 hr exposure at these concentrations resulted in 58% and 37% relative survival, respectively. The sharp inflexion of the dose-response curve for DMBA treatment (Fig. 3.3b) can probably be explained by the low

solubility of this compound in aqueous solvents. The limiting solubility of DMBA in F10 medium is, therefore, presumed to be 47 µg/ml.

The mutation of CHO-K1A cells to Oua^R was assessed using an *in situ* protocol. In this type of assay cells are plated, treated with a mutagen and then left undisturbed except for the addition of the selecting agent. The alternative respreading protocol, though less susceptible to interference from metabolic cooperation (1.7), should not be used to assess mutation to Oua^R. Trypsinisation, necessary for the dispersal of cells, has been reported to lead to the loss of newly arisen Oua^R mutants in respreading assays (Arlett, 1977a). The optimum conditions for the recovery of Oua^R CHO-K1A mutants, as validated by Dewdney (1982), are similar to those for other CHO-K1 cells (Hsie et al., 1981).

Consistent with the findings of Baker et al. (1974) and Dewdney (1982) EMS (Tables 3.4 and 3.10) was a far more effective inducer of viable Oua^R CHO-K1A mutants than MNNG (Tables 3.1 and 3.7). Conversely, MMS was a poor inducer of Oua^R CHO-K1A mutants (Tables 3.3 and 3.9). Similar observations have been reported for MMS in other Oua^R systems (Arlett et al., 1975; Bradley et al., 1981). In bacteria MMS mutagenesis has similarities to that of ionizing radiations (Bridges et al., 1973). Thus, an explanation for the low mutagenic efficiency of MMS at the Oua^R locus in mammalian cells can be derived from this radiomimetic property. Ionizing radiations, which cause deletions of whole genes and chromosomes, are also incapable of increasing spontaneous mutation to Oua^R in mammalian cells (Arlett, 1977a; Gupta and Siminovitch, 1980).

A weak mutagenic activity was observed for CEP and DMAE, while DMAP did not enhance spontaneous levels of Oua^R mutations in CHO-K1A cells (Table 3.5). The mutagenic activity of these compounds in the

CHO-K1/Oua^R system, therefore, correlates with their ability to form the active aziridinium ion (1.7.3). This is consistent with results obtained for these compounds in both bacterial (Soper et al., 1979) and mammalian cells (Thompson et al., 1981).

For DMBA, a weak mutagenic activity was detected at the Oua^R locus, in the absence of an exogenous activating system (Table 3.6). This confirms the observations from toxicity studies that CHO-K1A cells retain some endogenous metabolic activity for polycyclic hydrocarbons. A similar weak mutagenic response at the Oua^R locus has been reported for DMBA in CHO cells in the absence of exogenous activation (Gupta and Singh, 1982).

The effects of a single TPA concentration, 1 μg/ml, on spontaneous and chemical-induced mutation of CHO-K1A to Oua^R was examined using a standard protocol (Fig. 3.4). In these initial experiments cells were maintained in F10 medium. Cells were exposed to TPA from the end of mutagen treatment for the remainder of mutation expression and the whole of mutant selection. This protocol ensured that if the cells were susceptible in the mutagenesis enhancing activity of TPA for a particular period following treatment, as seemed probable from the observations of Dewdney (1982), the promoter would be present for that period.

TPA alone was neither toxic nor mutagenic at the Oua^R locus in CHO-K1A (3.3). Subsequent investigations showed a similar non-mutagenic response for TPA at other genetic loci in CHO-K1A cells (3.4 and 3.5). Furthermore, TPA did not enhance the lethal effects of mutagen treatment of CHO-K1A cells. These observations are in agreement with those obtained in CHO-K1A (Dewdney, 1982) and other mammalian cell systems (Lankas et al., 1977; Trosko et al., 1977; Thompson et al., 1980; Lasne et al., 1980).

In initial investigations (3.3.2) the effects of TPA on chemically-induced mutation to Oua^R were inconclusive. Each mutagen was tested at a single concentration yielding approximately 25% plating efficiency. At a similar survival level, TPA has been reported to enhance both MNNG- and MAM-induced mutation of V79 cells to Oua^R (Lankas et al., 1977). In CHO-K1A cells, however, this was not sufficient to elicit a TPA mutagenesis enhancing effect for any of the mutagens. Although TPA did increase the frequency of both MNNG- (Table 3.1) and MNU-induced (Table 3.2) mutation to Oua^R at this survival level, the increase was not significant.

That the concentration of the initiating mutagen may be important to the effects observed for TPA has been noted previously (Lankas et al., 1977; Dewdney, 1982). The simple alkylating agents, MNNG, MNU, MMS and EMS were therefore re-examined at concentrations yielding lower plating efficiencies (10 - 15%) in the same protocol (3.3.3). In this series of experiments the findings of Dewdney (1982) were confirmed. TPA enhanced the expression of MNNG-induced lesions (Table 3.7) but not lesions induced by EMS (Table 3.10) as Oua^R mutations in CHO-K1A cells. Differences in protocol which may have explained the results of Dewdney (1982) cannot be the reason for these present observations. TPA also enhanced the expression of MNU-induced (Table 3.8) but not MMS-induced (Table 3.9), mutation of CHO-K1A cells to Oua^R. The lesions enhanced by TPA in these cells therefore, appear to be those specific to the N-nitroso class of alkylating agents, rather than those common to all alkylating agents. As MNNG and MNU induce a similar pattern of DNA methylation (Lawley, 1974), presumably TPA enhances the mutagenesis of both agents by a common mechanism.

Preliminary experiments by Dewdney (1982), with CHO-K1A cells, suggested that there were differences in mutation enhancement by TPA at different genetic loci (Oua^R and TG^R). This may be similar to the organ specificity reported for tumour promoters *in vivo* (Table 1.1). If, as seems possible, the action of tumour promoters *in vitro* is locus specific, the study of their effects at other genetic loci may provide information as to the nature of the specific lesion(s) enhanced. The investigations of the effects of TPA on mutation induced by a single agent, MNNG, were therefore extended to other drug-resistance markers in CHO-K1A cells, TG^R (3.4), and Emt^R and $MGBG^R$ (3.5). The TG^R system has been well characterised for CHO-K1 cells (O'Neill et al., 1977; O'Neill and Hsie, 1979). Conversely, the multiple drug-resistance mutation assay described by Gupta and Singh (1982), for CHO cells, has not been characterised for CHO-K1 cells. However, with the ability to assess mutagenic effects simultaneously at independent genetic loci this may provide a more extensive screening system for compounds with tumour promoting activity.

Quantification of MNNG mutagenesis at the TG^R locus of CHO-K1A cells employed a respreading protocol based on that developed by O'Neill et al. (1977) for the CHO-K1 subclone BH_4. In an initial experiment (3.4.2) the observations of O'Neill et al. (1977), with respect to the phenotypic delay for induced TG^R mutants, were confirmed. For three concentrations of MNNG (0.04, 0.08, and 0.16 μg/ml) the maximal expression of TG^R mutation occurred 7-11 days after mutagen treatment (Fig. 3.6). However, two differences were apparent between CHO-K1A and CHO-K1-BH_4 cells. The time of maximal TG^R expression for CHO-K1A cells was dependent upon the MNNG concentration, and a decline in this peak mutation frequency was evident at all MNNG concentrations.

In contrast to the expression profiles for CHO-K1A cells (Fig. 3.6), O'Neill et al. (1977) reported that the expression time for TG^R CHO-K1-BH_4 mutants was independent of MNNG concentration. Also, there was no loss of mutants during subculture once maximal expression was attained in CHO-K1-BH_4 cells. The loss of TG^R CHO-K1A mutants during subculture suggests that these cells are at some selective disadvantage when compared to wild-type cells. A similar "peaked' expression profile has been reported for EMS-induced mutation of CHO-K1A cells to TG^R. The loss of TG^R mutants during subculture may, therefore, be a characteristic peculiar to CHO-K1A cells.

The effects of TPA on MNNG-induced mutation of CHO-K1A cells to TG^R were examined using two different promoter exposure regimes (3.4.3 and 3.4.4). When MNNG-treated cells were continuously exposed to TPA from 24 hr after the initiation of mutagen treatment (3.4.3) no enhancement of induced mutation was observed at any time (Table 3.5). However, these results did suggest that TPA may affect the expression profile of MNNG-induced TG^R CHO-K1A mutants. The previously observed peaked profile, with maximal TG^R recovery at day 9, was replaced by a plateau of induced TG^R mutants recovered between days 7 and 11 for TPA-treated cells.

The effect of TPA on MNNG-induced TG^R mutation are in contrast to those obtained previously for EMS-induced TG^R mutations in CHO-K1A cells. Using a similar promoter exposure, a significant enhancement of EMS-induced TG^R mutation was observed on days 6, 8 and 11 of expression (Dewdney, 1982).

In a more complete study (3.4.4), cells were exposed to TPA from the end of the effective mutagen treatment time (2 hr, O'Neill et al., 1977) for the remainder of mutation expression and the whole of mutant selection. The results in Table 3.16 (illustrated in Fig. 3.7)

show that TPA enhanced the early expression of MNNG-induced TG^R CHO-K1A mutants. A significant enhancement of the incidence of MNNG-induced TG^R mutants was observed on days 5 and 7 following TPA treatment. As TPA does not affect the growth parameters of CHO-K1A cells (Table 2.4), the early expression of TG^R mutants is not simply due to an increased rate of HGPRT loss from newly induced TG^R mutants. TPA also affected the expression profile of TG^R mutants. Although not significantly higher than for MNNG alone on days 9 and 11, and recovery of TG^R mutants was maintained in TPA exposed cells. That is, there was no loss of TG^R mutants during subculture once maximal expression frequency was attained.

MNNG mutagenesis at the Emt^R, $MGBG^R$ and Oua^R loci in CHO-K1A cells was assessed using the replating method of Gupta and Singh (1982) (3.5.1). The incidence of spontaneous Emt^R and $MGBG^R$ CHO-K1A mutants was comparable with that reported for CHO cells (Gupta and Singh, 1982). MNNG mutagenesis has yet to be assessed in CHO cells at these loci using this mutation assay, however, other alkylating agents have been found to induce Emt^R, $MGBG^R$ and Oua^R mutants (Gupta and Singh, 1982).

Although the numbers of viable mutants was small, compared with results obtained in other mutation assays, MNNG was mutagenic at these three genetic loci in CHO-K1A cells (Table 3.17). The incidence of MNNG-induced $MGBG^R$ CHO-K1A mutants was comparable to that induced in CHO cells by other alkylating agents. However, the incidence of MNNG-induced Emt^R CHO-K1A mutants was higher than those observed in CHO cells (Gupta and Singh, 1982). This was probably explained by an insufficient concentration of Emt in the selection medium in this experiment (3.5.2).

When compared to the results obtained using the *in situ* protocol

(Table 3.7), the incidence of MNNG-induced Oua^R CHO-K1A mutants is considerably reduced in this replating assay (Table 3.17). This is probably because the newly arisen Oua^R mutants, known to be sensitive to trypsinisation, are lost during subculture. Additionally, in this mutation assay Oua^R mutants are selected following an expression time of 72 hr, while the optimum expression time for Oua^R CHO-K1A mutants is 48 - 52 hr (Dewdney, 1982). Once this optimum expression period is exceeded the recovery of Oua^R mutants has been reported to decline in CHO-K1A cells (Dewdney, 1982).

The mutagenesis enhancing activity of TPA, examined at these three genetic loci (3.5.3), confirmed the locus specific action of this tumour promoter *in vitro*. The expression of MNNG-induced mutation of CHO-K1A cells to Emt^R, but not to $MGBG^R$ was enhanced by TPA (Table 3.19). These results also suggested that TPA may exert a cytotoxic effect on newly arisen Oua^R cells.

The results obtained in the multiple drug-resistance marker system should, however, be considered in the light of other observations.

1) The multiple drug-resistance marker mutation assay has yet to be validated for CHO-K1A cells. The selection conditions for CHO resistance mutants in this assay system (Gupta and Singh, 1982) are not optimal for Oua^R CHO-K1A mutants (Dewdney, 1982), and by analogy, may not be optimal for the recovery of either Emt^R or $MGBG^R$ CHO-K1A mutants. Indeed, an Emt concentration of 1.5×10^{-7} M, sufficient for the selection of Emt^R CHO mutants, was insufficient to kill wild-type CHO-K1A cells within the period of mutant selection (3.5.1).

For Emt^R CHO-K1A mutants to be accurately scored the concentration of Emt was increased to 2.5×10^{-7} M (Table 3.18). Modifications

of other selection parameters might also be required.

2) The experimental protocol for this assay (Fig. 3.8) is very cumbersome, requiring a great number of individual manipulations of the cultures. Thus, the system may be susceptible to interferences from external factors. For example, trypsinisation of the cultures immediately prior to mutant selection may affect the recovery of mutants at some loci on subsequent exposure to tumour promoting agents. This may provide an explanation for the cytotoxic effect of TPA to newly arisen Oua^R mutants (Table 3.19). As TPA is a membrane active agent,this may be a consequence of the additional stresses placed on the membranes of Oua^R cells, already selectively disadvantaged by trypsinisation. This apparent interaction between trypsinisation and TPA may also affect the recovery of TG^R mutants in the respreading-type protocol used in 3.5. However, there is no evidence to suggest that this is important to the results obtained for TPA in the CHO-K1/TG^R system.

Of the different mutation assay systems examined the Oua^R system (3.3) appeared to be the most valuable for use in subsequent studies. It provided a fully validated protocol for CHO-K1A cells in which a mutagenesis enhancing activity for TPA could be reliably detected when either MNNG or MNU was used as the initiating mutagen. It also offered other advantages to mutagenesis enhancing activity studies. Oua^R mutations were assessed in an *in situ* protocol, which is less susceptible to external influences such as trypsinisation, and less manipulation of the cells was required.

For these reasons this Oua^R system was used to investigate the mechanism of *in vitro* mutagenesis enhancement by tumour promoters in CHO-K1A cells and other Chinese hamster cell lines.

Cell culture conditions have been reported to influence the *in vitro* activity of TPA (Nagasawa and Little, 1981). They have also been

implicated to explain the discordant results obtained for TPA by different laboratories (Emerit and Cerutti, 1981). With growing evidence for the involvement of free radicals in tumour promotion, the free-radical scavenging potential of cell culture medium has been of interest. By examining the influence of culture conditions on the mutagenesis enhancing activity it was hoped to obtain an indication of the mechanism(s) of this enhancing activity in CHO-K1A cells and other cell lines. The influence of free radical scavenging agents in the culture medium on mutagenesis enhancing activity of TPA and other promoters was examined using the Oua^R system.

The sulphydryl compounds L-cysteine and glutathione (GSH) have been shown to protect cells in culture from the DNA damaging effects of X-rays and radiomimetic chemicals (Sasaki and Matsubara, 1977; Raj and Heddle, 1980). Glutathione also has an important role in the intracellular defence against free radicals produced during normal metabolic processes and at times of oxidative stress (Meister and Anderson, 1983).

Cell culture conditions had a profound influence on the mutagenesis enhancing activity of tumour promoters in CHO-K1A cells. For TPA (4.3), the presence of L-cysteine was essential to the enhancing activity of this agent on MNNG-induced mutation of CHO-K1A cells (Tables 3.7, 4.2, and 4.3b). When L-cysteine was absent from the culture medium (Tables 4.1 and 4.3a) or replaced by GSH (Table 4.4) TPA had no effect on MNNG mutagenesis. This absolute requirement for L-cysteine is in contrast to the results of Perchellet et al. (1985). These workers have found that the addition of L-cysteine (4×10^{-4} M) to the culture medium of mouse keratinocytes prevented the induction of ODC activity associated with TPA treatment of these cells. The mutagenesis enhancing activity of TPA was mutagen specific, confirming previous observations from 3.3.3. TPA did not enhance EMS-induced mutation of CHO-K1A cells to Oua^R,

irrespective of culture conditions (Tables 3.10 and 4.5). Culture conditions did not influence the cytotoxicity or mutagenicity of TPA alone, or the effects of TPA on mutagen-induced lethality.

In contrast, a mutagenesis enhancing activity could only be detected for the free radical generating compound BZP in the absence of L-cysteine, for cells grown in EMEM (4.4). This is consistent with this medium having the lowest free radical scavenging potential of those studied (Keck and Emerit, 1979). Thus, a free radical mechanism of mutagenesis enhancement can be detected by alterations of culture conditions. As would be expected for this type of mechanism of mutagenesis enhancement the activity of BZP was not mutagen specific. In the absence of L-cysteine, BZP enhanced both MNNG- (Table 4.8) and EMS-induced (Table 4.10a) Oua^R mutation in CHO-K1A cells. In these experiments, and essential to its definition as a true mutagenesis enhancing agent, BZP at a concentration of 1.5 µg/ml was neither toxic to control or mutagen-treated CHO-K1A cells, nor mutagenic at the Oua^R locus.

The small, though not significant, increase of MNNG- and EMS-induced Oua^R mutations of CHO-K1A cells grown in EMEM + LC (Tables 4.9 and 4.10b) by BZP may be due to insufficient protection of the cells by a concentration of 2×10^{-4} M L-cysteine. In F10 medium, a more complex cell culture medium, MNNG mutagenesis was not increased by BZP (Table 4.7).

At a non-toxic and non-mutagenic concentration, 0.6 µg/ml, HCHO exhibited a similar profile of MNNG-mutagenesis enhancement as observed for TPA. A mutagenesis enhancing activity for HCHO was only detected when L-cysteine was present in the culture medium (Table 4.11). This suggests that there may be similarities in the mechanism of mutagenesis enhancement for both TPA and HCHO.

In CHO-K1A an *in vitro* mutagenesis enhancing activity could not be detected for the linear alkanes n-decane (Table 4.13), n-dodecane (Table 4.14) or n-tetradecane (Table 4.15), irrespective of culture conditions. This is in contrast to their reported mutagenesis enhancing activity in V79 cells (Lankas et al., 1978). The linear alkanes may act as tumour promoters by altering membrane functions (Horton et al., 1976), and a similar mechanism may account for their mutagenesis enhancing activity in V79 cells. By comparing the results obtained for the linear alkanes and TPA in CHO-K1A cells it can be inferred that the effects of TPA on MNNG mutagenesis are not simply due to a general perturbation of membrane activity.

The *in vitro* mutagenesis enhancing activity of the linear alkanes in V79 cells parallels their *in vivo* tumour promoting activity in mouse skin (Sice, 1966). This has been used to support the proposal that the detection of *in vitro* mutagenesis enhancing activity may provide a much-needed rapid screening test for compounds with tumour promoting properties (Lankas et al., 1980). However, it is apparent from these present studies that the *in vitro* mutagenesis enhancing activity of promoters in CHO-K1A cells does not parallel their *in vivo* promoting activity. These present studies also indicate that promoting agents may exhibit cell line specificity for their *in vitro* activities, similar to the organ and species specificity observed for these agents *in vivo* (Table 1.1).

This cell line specificity of tumour promoters was examined using other Chinese hamster cell lines. For both V79 and CHO cells mutation to Oua^R can be quantified using exactly the same *in situ* protocol as that used for CHO-K1A cells (Bradley et al., 1981; Hsie et al., 1981). Any results obtained can therefore be directly compared to the observations in CHO-K1A cells.

Prior to mutagenesis studies in these cell lines the cytotoxicity of the mutagens to be used was assessed. Both V79-379A and CHO(S) cells were extremely sensitive to the cytotoxic effects of MNNG when treated as single cells. Depending upon the culture medium the D_{37} value for MNNG in V79-379A cells ranged from 0.011 µg/ml - 0.03 µg/ml (Figs. 5.1a and 5.1b). The reported D_{37} values for other V79 cells obtained under similar conditions are 0.74 µg/ml (Chu et al., 1968) - 1.03 µg/ml (Peterson et al., 1979). For CHO(S) the D_{37} value for MNNG was 0.011 µg/ml (Fig. 5.2) compared to the value of 0.44 µg/ml obtained by Kao and Puck (1968). The reason(s) for the extreme sensitivity of these cells remains to be elucidated. However, it was not due to the degradation or contamination of the MNNG used throughout these studies. The UV absorption spectrum for the MNNG used throughout showed no observable differences from that of a newly acquired batch of MNNG (see Appendix).

The toxicity of EMS towards single-plated V79-379A cells was also assessed (Fig. 5.1c). The D_{37} value for EMS treatment was 0.49 mg/ml. This compares to values of 1.5 mg/ml and 1.2 mg/ml obtained by Arlett et al. (1975) and Peterson et al. (1979), respectively, for V79 cells under similar conditions. V79-379A cells are therefore more sensitive to the cytotoxic effects of both MNNG and EMS than CHO-K1A cells treated under similar conditions.

In initial mutagenesis experiments TPA (1 µg/ml) seemed to be toxic to both control and MNNG-treated V79-379A cells (Tables 5.1 - 5.3), under all culture conditions. This is in contrast to the results of other workers using V79 cells (Lankas et al., 1977, 1980; Trosko et al., 1977), and may have been caused by initial handling problems encountered with this cell line. The trypsinisation conditions were very difficult to

establish for V79-379A cells and different requirements were necessary for cells grown in different culture media (see Table 2.2). In a subsequent experiment TPA was not toxic to either control or EMS-treated V79-379A cells (Table 5.4).

In agreement with the results of Thompson et al. (1980) TPA was not toxic to either control or MNNG-treated CHO(S) cells (Table 5.5). TPA alone was not mutagenic at the Oua^R locus in either V79-379A or CHO(S) cells.

In agreement with the observations of Lankas et al. (1977), and despite its apparent cytotoxic effects, TPA did enhance gene mutation at the Oua^R locus in V79-379A cells. In contrast to the results observed in CHO-K1A cells under similar conditions, this mutagenesis enhancing activity of TPA in V79-379A cells was neither mutagen specific nor dependent upon culture conditions (5.2). TPA enhanced both MNNG- (Tables 5.1 - 5.3) and EMS-induced (Table 5.4) Oua^R mutation of V79-379A cells. Furthermore, the presence of L-cysteine was not required for this enhancing activity on MNNG mutagenesis. This suggests that the mechanism of mutagenesis enhancement by TPA in CHO-K1A and V79-379A cells may be different and cannot be explained by the use of different experimental protocols.

In CHO(S) cells TPA did not enhance MNNG-induced mutation to Oua^R, despite the presence of L-cysteine in the culture medium (Table 5.5). The lack of a mutagenesis enhancing activity for TPA in this cell line is in agreement with that reported by Thompson et al. (1980) in CHO cells. However, it is possible that this lack of activity may be due to a mutagen concentration effect previously observed in CHO-K1A cells (3.3).

It has been proposed that the mutagenesis enhancing activity of tumour promoters in mammalian cells *in vitro* is an experimental artefact

(Lasne et al., 1980; Thompson et al., 1980; Kinsella, 1981). This stems from a number of observations that promoting agents inhibit cell-cell communication between cells in culture. The first evidence that tumour promoters could inhibit intercellular communication was obtained independently by Yotti et al. (1979) and Murray and Fitzgerald (1979). Since which time promoter-mediated inhibition of cell communication has been observed in many types of cultured cells, including human cells, by means of metabolic cooperation (Trosko et al., 1982; Lawrence et al., 1984), electrical coupling (Enomoto et al., 1981; Yamasaki et al., 1983) and dye transfer methods (Fitzgerald et al., 1983; Enomoto and Yamasaki, 1985a).

Of particular importance to the *in vitro* mutagenesis enhancing activity of promoters are the observations that at very low concentrations TPA and other promoting agents enhanced the recovery of TG^R V79 cells cocultured with high densities of wild type (TG^S) cells in selective medium (Yotti et al., 1979; Trosko et al., 1982). At the TG^S cell densities used, in the absence of the promoters, the TG^R cells were rendered phenotypically normal as a result of metabolic cooperation and therefore unselectable in TG-containing medium.

Between cells in contact metabolic cooperation is thought to be mediated through specialised membrane structures, gap junctions (Gilula et al., 1972; Azarina et al., 1974). These are the likely sites for the transfer of ions, metabolites and other molecules between normal and mutant cells. For V79 cells, freeze fracture studies have demonstrated that TPA almost completely eliminated existing gap junctions, and prevented the formation of new structures (Yancey et al., 1982).

The mutagenesis enhancing activity for TPA observed in CHO-K1A cells is unlikely to result from an inhibition of metabolic cooperation. In

the Oua^R system this was established by a number of observations. Neither the mutagen specificity of the enhancing activity (3.3) nor the profound influence of the culture conditions (4.3) would be expected if TPA or the other tumour promoters exerted their effects in this manner in this cell line. In addition, the enhancing activity of TPA increased with a decrease in CHO-K1A cell survival, when lower densities of cells would be expected at mutant selection.

The results of a reconstruction experiment for CHO-K1A cells (3.3.5) showed that the colonial recovery of Oua^R cells in the presence of 2×10^5 wild-type cells increased by approximately 20% in the presence of either TPA or DMSO. However, a similar increase (approximately 16%) in the recovery of Oua^R colonies in the presence of untreated wild-type cells, over that in their absence, has been reported previously for CHO-K1A cells (Dewdney, 1982). This was presumed to reflect a feeder layer effect whereby a large number of wild-type cells facilitated the colonial growth of Oua^R cells. Neither TPA nor DMSO interfere with this form of cellular communication.

It is also unlikely that inhibition of metabolic cooperation can account for the enhancement in the incidence of TG^R mutants at days 5 and 7 of expression following TPA exposure of MNNG-treated CHO-K1A cells (Fig. 3.7). In reconstruction experiments performed independently with two different CHO-K1 subclones it has been shown that when cells are respread in TG-containing medium at less than 3×10^5 cells/90 mm dish cell density had no effect on the recovery of TG^R mutants (Chasin, 1973; O'Neill et al., 1977). As in the TG^R experiments reported above (3.4) the cell density was 1.5×10^5 cells/90 mm dish the possibility of metabolic cooperation was therefore assumed to be eliminated.

CHO, the parental cell line of CHO-K1, has only a limited capacity for metabolic cooperation (Corsaro and Migeon, 1975). This implies that

CHO-K1 cells likewise do not engage in this form of intercellular communication to any great extent. The absence of TPA mutagenesis enhancing activity in CHO(S) cells reported in these present studies (5.3.2) further supports a mechanism other than an inhibition of metabolic cooperation to explain the effects observed in CHO-K1A cells. Interestingly, TPA has been reported to slightly enhance metabolic cooperation between TG^R CHO mutants and high densities of wild-type CHO cells (Warren et al., 1981). This may explain the slight, though not significant, decrease in MNNG-induced mutation of CHO(S) cells to Oua^R in the presence of TPA (Table 5.6).

In contrast to CHO cells, metabolic cooperation has been repeatedly demonstrated between normal and mutant V79 cells (Yotti et al., 1979; Warren et al., 1981; Trosko et al., 1982). Thus, the mutagenesis enhancing activity of tumour promoters in V79 cells could result from an inhibition of metabolic cooperation. The results observed for V79-379A cells in this present study (5.2) support this as the probable mechanism of action of TPA in this cell line. The mutagenesis enhancing effect of TPA in V79-379A cells was neither mutagen specific nor dependent upon culture conditions. Also in a reconstruction experiment (5.2.4) TPA enhanced the recovery of Oua^R colonies in the presence of wild-type V79-379A cells (Table 5.5). This inhibition of metabolic cooperation by TPA was almost complete, as the recovery of Oua^R colonies was similar in the presence or absence of Oua^S V79-379A cells following TPA treatment (56.2% v 63%, Table 5.5). Similar results are reported for TPA enhancement of TG^R mutant recovery in the presence of high densities of TG^R V79 cells (Yotti et al., 1979). These observations are in agreement with those of Yancey et al. (1982) that TPA almost completely eliminates gap junctions in V79 cells. Differences in the ability of

CHO-K1 and V79 cells to engage in metabolic cooperation may explain the contrasting mutagenesis enhancing activity of the linear alkanes in these cell lines.

Although considered an experimental artefact *in vitro*, the inhibition of cell-cell communication by tumour promoters may be relevant to their *in vivo* promoting activity. The ability to inhibit metabolic cooperation between TG^R and TG^S V79 cells has been correlated with *in vivo* tumour promoting ability (Trosko et al., 1982). In addition, Kalimi and Sirsat (1984) have recently reported a decreased number of gap junctions in TPA-treated mouse skin. As many malignant tumours *in vivo* are deficient in gap junctions (Weinstein et al., 1976), these observations suggest that promoter-mediated inhibition of cellular communication may permit the release of initiated cells from normal growth restraints. That the synthetic diacylglycerol, OAG (see 1.6), also inhibits metabolic co-operation (Enomoto and Yamasaki, 1985b) implies that this effect of phorbol diesters may be mediated through binding with their specific membrane receptors.

While the mutagenesis enhancing activity of TPA in V79-379A cells may be an "experimental artefact", the enhancing activity of TPA in CHO-K1A cells appears to be a real effect. However, for this cell line some mechanism of action must explain the mutagen specificity (3.3) and the influence of culture conditions (4.3). Three genuine mechanisms, distinct from effects on metabolic cooperation, have been suggested which might explain the mutagenesis of enhancing activity of tumour promoters:

1) induction of the expression of latent mutations;

2) inhibition of DNA repair; and

3) disturbances of oxidative metabolism.

The induction of latent mutations by TPA is less mechanistically specific than the other two. This hypothesis envisages that not all mutagen-

induced primary DNA lesions are expressed as mutations and that TPA and other tumour promoters act to activate these unexpressed mutations. This hypothesis is compatible with the characteristic capability of TPA to enhance mutation long after the occurrence of DNA damage. However, in CHO-K1A cells the enhancing activity of TPA was dependent on the early presence of the tumour promoter (3.3.4), which eliminates this as the mechanism of action of TPA in this system.

DNA repair is a mechanism by which a cell protects itself against mutagenic agents. The activated forms of most mutagens are capable of interacting covalently with cellular DNA (Miller, 1970) and most normal cells have enzyme systems to remove these bound mutagens (Hart et al., 1978). Short sections of single-stranded DNA containing lethal or mutagenic lesions can be repaired by an error-free excision mechanism. During this, damaged DNA is excised, by the actions of endonucleases and exonucleases, and then replaced with the correct sequence of nucleotides, by a process involving DNA polymerase and ligase activities. In mammalian cells this error-free excision repair process is detectable by the removal of pyrimidine dimers from DNA or the occurrence of unscheduled DNA synthesis (UDS) in synchronised cell populations (Auerbach, 1976). Studies with bacterial and mammalian cell mutants have demonstrated that an absence of, or a reduction in DNA excision repair capacities leads to enhanced mutagenesis (Witkin, 1969; Hart et al., 1978). Defective repair of damaged DNA has been related to carcinogenesis with the finding that cells from patients with the hereditary "cancer-prone" disease Xeroderma pigmentosum demonstrate a virtual absence of UDS following UV radiation (Setlow et al., 1969).

Gaudin et al. (1971, 1972) observed that TPA inhibited DNA excision repair in human lymphocytes following 254 nm UV irradiation.

Similarly, TPA has been shown to inhibit the removal of pyrimidine dimers from the DNA of UV (254 nm) irradiated HeLa cells, and to augment UV inactivation of this cell line (Teebor et al., 1973). In human amnion cells TPA also inhibits UV-induced UDS (Trosko et al., 1975). Thus, it may be that tumour promoters act to enhance mutagenesis by an inhibition of excision repair of mutational and/or premutational lesions in damaged DNA.

The possibility that the inhibition of excision repair, or other DNA repair processes, is responsible for the enhancement of mutagenesis by tumour promoters has been cast in to doubt.TPA was able to elicit increases in both TG^R and Oua^R mutation frequencies in V79 cells for up to 3 weeks after mutagen treatment (Trosko et al., 1977; Lankas et al., 1980). DNA repair in mammalian cells is essentially complete within the first 24 hr following the occurrence of DNA damage (Trosko and Yager, 1974).

In CHO-K1A cells, however, an inhibition of DNA repair may, in part, explain the mutagenesis enhancing activity of TPA, which was dependent on the duration of the promoter treatment (3.3.4). TPA was only effective as a mutagenesis enhancing agent when presented to MNNG-treated cells during the first 15 hr of mutation expression (Table 3.12). This is the time during which the majority of DNA repair (70 - 90%) in mammalian cells is effected (Edenberg and Hanawalt, 1973; Trosko and Yager, 1974).

The inhibition of DNA repair need not be a mechanism of mutagenesis enhancement exclusive to the tumour promoters. The non-promoting DNA repair inhibitors caffeine and 3-aminobenzamide have been reported to enhance induced-mutations in mammalian cell assays (Roberts and Sturrock, 1973; Schwartz et al., 1985). However, these are non-specific

DNA repair inhibitors, and also enhance the cytotoxicity of mutagens in these systems. As would be expected if the promoter had a non-specific effect on DNA repair, TPA enhanced the inactivation of CHO-K1A cells following exposure to 313 nm (Fig. 6.4) and 365 nm UV (Fig. 6.5). The importance of DNA damage to the lethal effects of these wavelengths is uncertain. At a wavelength where DNA damage is known to be of prime importance to cell inactivation (254 nm) TPA did not enhance UV-induced lethality (Fig. 6.3). Also, TPA did not enhance the cytotoxic effects of any of the mutagens in CHO-K1A cells (3.3.2 and 3.3.3). Thus, the effect of TPA is specifically directed at inhibition of the repair of mutational or premutational DNA lesions.

There is accumulating evidence from *in vivo* and *in vitro* studies that active oxygen species play a role in tumour promotion (Cerutti, 1985). Exogenously generated oxygen free radicals have also been reported to enhance *in vitro* transformation of initiated C3H/10T½ cells (Zimmerman and Cerutti, 1984) and to possess weak mutagenic and clastogenic activities in CHO cells (Phillips et al., 1984; Sofuni et al., 1984). DNA damage caused by induced free radicals may be reflected *in vitro* as a mutagenesis enhancing activity.

A direct free radical mechanism of action for the mutagenesis enhancing activity of TPA in CHO-K1A cells seems improbable. L-cysteine, present in culture medium, protects CHO-K1A cells against the enhancing activity of the free radical generating compound BZP (Tables 4.7 - 4.10). Conversely, the presence of L-cysteine is specifically required for the mutagenesis enhancing activity of TPA to be observed (4.3). Also, a direct free radical mechanism of mutagenesis enhancement should not be mutagen specific. As BZP enhanced the incidence of both MNNG (Table 4.8) and EMS-induced (Table 4.10) Oua^R mutations in CHO-K1A cells, this

further supports a direct free radical mechanism of mutagenesis enhancement for BZP.

That the mechanism of mutagenesis enhancement in CHO-K1A by BZP and TPA are different is in agreement with recent observations *in vivo* and *in vitro*. Reiners et al. (1984) have found that strains of mice refractory to promotion by TPA responded to BZP promotion, indicating that BZP may possess a more general promoting activity than TPA. While in cultured JB6 mouse epidermal cells retinoic acid, the potent inhibitor of Stage II tumour promotion (see 1.4), inhibits BZP-induced transformation but not that induced by TPA (Gindhart et al., 1985). In this study the range of concentrations, 10^{-9} M - 10^{-5} M, over which BZP induced transformation included the concentration employed in CHO-K1A studies (4.4).

However,the involvement of free radicals in the mutagenesis enhancing activity of TPA cannot be entirely eliminated. It has been proposed that the free radicals, known to be produced by cells in response to TPA treatment, do not induce genetic damage directly, but mediate their effects by an indirect mechanism (Emerit and Cerutti, 1981, 1982). A clastogenic factor, comprised of free arachidonic acid (AA) together with peroxides and aldehydes, produced by the action of free radicals on cellular membranes, is imperative to this mechanism (Fig. 7.1). It is the DNA damage induced by this clastogenic factor which may be manifest as mutagenesis enhancement.

There are similarities, with respect to culture conditions, between the mutagenesis enhancing activity of TPA (4.3) and HCHO (4.5). It appears that the aldehydic component of a clastogenic factor induced in CHO-K1A cells could be important to the mutagenesis enhancing activity of TPA.

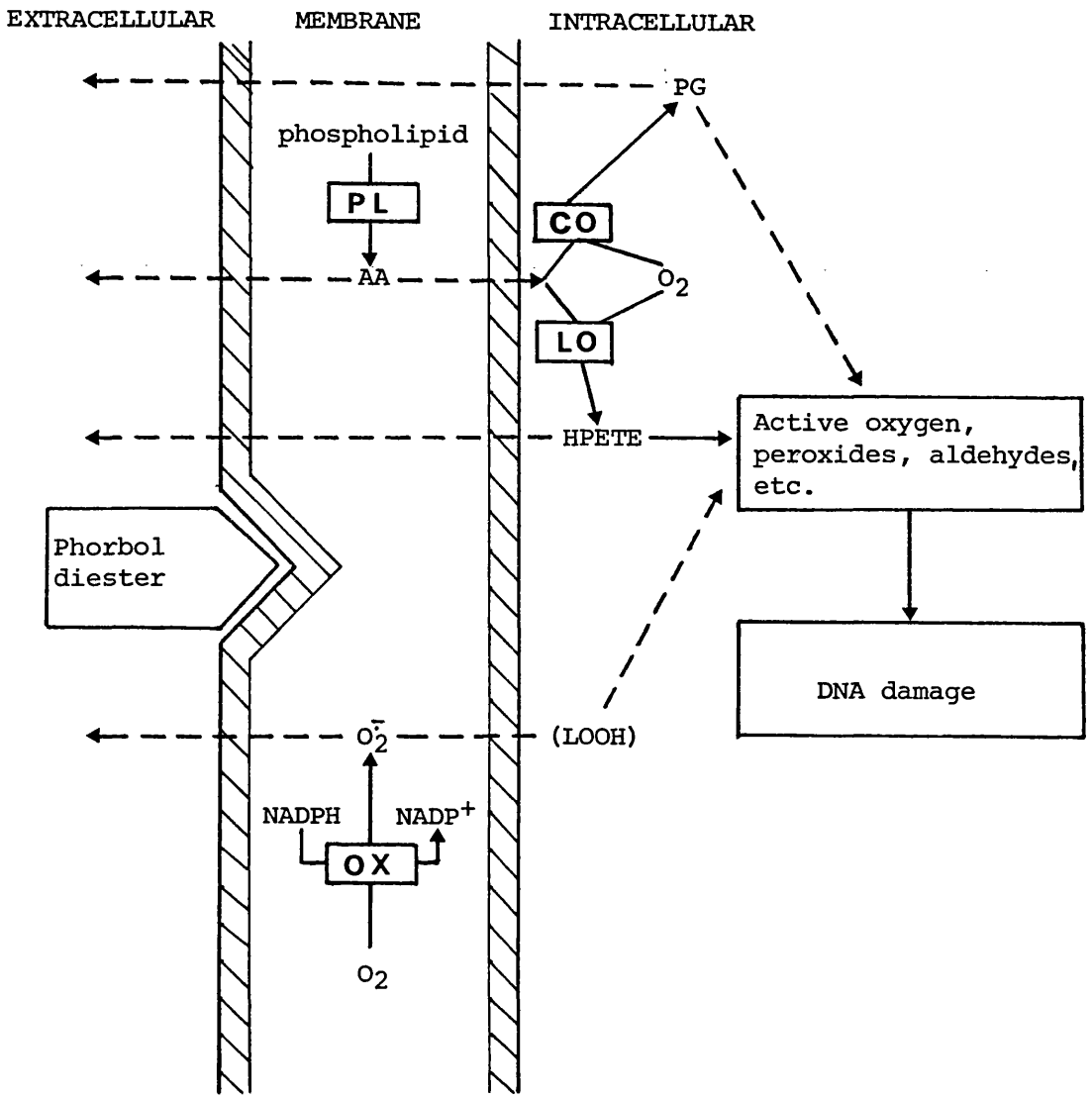


Fig. 7.1 Model of membrane mediated DNA damage.

Phorbol diesters can stimulate the arachidonic acid (AA) cascade and *via* the prostaglandins (PG) and the AA-hydroperoxides (HPETE) produce active oxygen and aldehydes. Alternatively, they can produce active oxygen and lipid hydroperoxides (LOOH) by an oxidative burst. Extracellular lipid hydroperoxides plus aldehydes may act as a clastogenic factor causing DNA damage directly in the stimulated cell or indirectly in the surrounding tissue.

CO cyclooxygenase, LO lipooxygenase, OX oxidase, PL phospholipase.

(Adapted from Cerutti and Amstad, 1983)

The mutagenesis enhancing activity of HCHO has been investigated in human bronchial epithelial cells (Grafstrom et al., 1985). In that system HCHO enhanced MNU mutagenesis by specifically inhibiting the action of O^6 methylguanine-DNA-methyltransferase (MT, E.C. 2.1.1.66). This is the repair enzyme present in many bacterial and mammalian cells responsible for the removal of the premutagenic O^6 methylguanine (O^6MeG) lesion induced by MNU and other alkylating agents (Samson and Cairns, 1977; Craddock et al., 1982). HCHO could inhibit the repair of O^6MeG by several different mechanisms. MT has a cysteine moiety at its active site (Pegg, 1983) and aldehydes readily bind to cysteine. Aldehydes such as malondialdehyde, formed by lipid peroxidation during clastogenic factor formation (Fig. 7.1) could also act to inhibit the removal of O^6MeG by binding to the active site of MT (Grafstrom et al., 1985).However, as CHO cells do not possess MT activity (Goth-Goldstein, 1980), this cannot explain the mechanism of mutagenesis enhancement by either HCHO or TPA. Alternatively, HCHO and other aldehydes react directly with media components such as cysteine to produce cyclic compounds (Fig. 7.2). Similar cyclic compounds have been reported to inhibit enzyme functions in bacteria (Kay and Gronhund, 1969), and Grafstrom et al. (1985) have proposed that they may also inhibit DNA repair enzymes in mammalian cells. This would explain the influence of culture conditions on the mutagenesis enhancing activity for both HCHO (4.5) and TPA (4.3) in CHO-K1A cells. Such an inhibition of DNA repair functions would also explain the influence of the duration of promoter treatment on the activity of TPA in these cells (3.3.4). The mutagen concentration should be important in such a mechanism, as a higher dose of mutagen will induce greater numbers of premutagenic lesions to be excised from DNA. TPA activity in CHO-K1A cells exhibits such a dependency on the dose of the initiating mutagen (3.3.2 and 3.3.3).

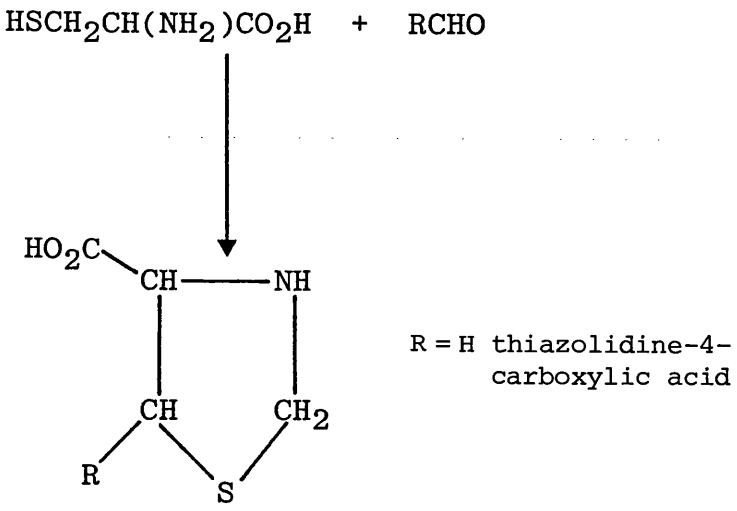


Fig. 7.2 Reaction of cysteine with aldehydes producing thiazolidine compounds.

(Taken from an oral presentation given by R.C. Grafstrom to the Fourth International Conference on Environmental Mutagens, Stockholm, 1985.)

The nature of the DNA lesion whose repair is inhibited during TPA treatment may be inferred from the mutagen specificty of this activity. In CHO-K1A cells TPA specifically enhanced the mutagenesis of MNNG and MNU (3.3.3) which indicates that an O-alkylation lesion in DNA may be important in this process. S_N1 alkylating agents (see 1.7.2), typified in this present study by MNNG and MNU, react relatively more extensively at O-atom sites in DNA than do S_N2 agents e.g. MMS (Lawley, 1974). The borderline $S_N1/2$ compound EMS fills an intermediate role, but does not appear to produce the specific lesion enhanced by TPA in sufficient amounts for this activity to be exhibited.

The induction of O^6 alkylG in DNA has been correlated with the mutagenicity of alkylating agents in mammalian cells (Heflich et al., 1983; Kaina et al., 1983). However, there is evidence to suggest that at least one other DNA lesion, O^4 alkylthymine (O^4MeT), may also be responsible for some of the mutagenic effects of alkylating agents (Abbott and Saffhill, 1977; Fox and Brennand, 1980). An inhibition of the repair of this minor DNA lesion may explain the effects of TPA.

There is no direct evidence to indicate that CHO cells possess a repair enzyme for O^4MeT excision. However, although they do not possess MT activity, CHO cells have been shown to exhibit an inducible adaptive response (Samson and Schwartz, 1980), similar to that associated with MT activity in bacteria (Samson and Cairns, 1977). Waldstein et al. (1982) have also reported a limited capacity for MNNG-treated CHO cells to remove O-methyl lesions from their DNA. The presence of an inducible O^4MeT repair enzyme has been implicated to explain similar adaptation responses in V79 cells (Fox and Carlton,1984), which also lack MT activity (Warren et al., 1979). Thus it is possible that CHO-K1A cells may possess an O^4MeT repair enzyme.

This mechanism of mutagenesis enhancement is dependent on the production of a clastogenic factor in CHO-K1A through an interaction between TPA and cell membranes (Fig. 7.1). Two observations suggested that these effects of TPA could be membrane mediated in CHO-K1A cells:

a) TPA induced changes in the morphology of CHO-K1A cells, although it did not affect other growth parameters (2.8.10); and

b) in the multiple drug-resistance marker assay (3.5) TPA was toxic to Oua^R CHO-K1A mutants (Table 3.19). Oua^R mutants are known to be sensitive to the membrane activity of trypsin (Arlett, 1977a).

Additionally, TPA is a membrane active compound (see 1.2), and it is becoming increasingly apparent that a large number of biological effects of TPA can be mediated through interactions with cell membranes (see 1.6). The effects of TPA on the inactivation of CHO-K1A cells following UV irradiation (Chapter 6) further support a membrane activity of TPA in these cells.

From action spectra studies with cultured cells, it is apparent that the lethal effects of UV radiations are mediated by different lesions at different wavelengths (Jagger, 1985). In the far-UV region (< 290 nm) DNA damage is almost certainly responsible for cell inactivation (Coohill, 1984). In this UV region the cytotoxicity of the radiations correlates with the induction of DNA photoproducts, most particularly pyrimidine dimers, for a number of cell culture systems (Todd et al., 1968; Rothman and Setlow, 1979). While DNA lesions are undoubtedly involved in cell inactivation by mid- (290-320 nm) and near-UV (320-400 nm) radiations (Webb, 1977; Jagger, 1981), it is apparent that damage to other cellular components is also of importance. Studies in bacteria (Moss and Smith, 1981; Kelland et al., 1983a, 1984) and in yeast (Ito and Ito, 1983) have suggested that the cell membrane may be the target site for some of the

lethal damage induced by these UV radiations. This was concluded because following mid- and near-UV irradiation cells either became more sensitive to other membrane damaging events (Moss and Smith, 1981; Kelland et al., 1983a) or leaked essential intracellular components into their culture media (Ito and Ito, 1983; Kelland et al., 1984). The former rationale was used to assess the membrane activity of TPA, by exposing CHO-K1A cells irradiated with far-, mid- and near-UV to TPA (1 μg/ml) in the plating medium (6.3). TPA enhanced cell inactivation by 313 nm (Fig. 6.4) and 365 nm (Fig 6.5) UV but not that following 254 nm UV (Fig. 6.3), while having no effect on unirradiated cells. The lack of effect at 254 nm is in contrast to that of Dewdney (1982), who found that TPA mildly sensitized CHO-K1A cells to inactivation by 254 nm UV. However, in that study the cells were trypsinised immediately prior to TPA exposure, which may explain their sensitivity.

Although in all experiments the cells were irradiated under essentially the same conditions, the irradiation times in the 313 nm and 365 nm experiments were considerably longer than those at 254 nm. The observed differences could therefore have been due to the prolonged holding of CHO-K1A cells in PBS, common to 313 nm and 365 nm experiments. When this was investigated in a sham-irradiation experiment (6.4), TPA had no effect on the survival of non-irradiated CHO-K1A cells held for up to 210 mins in PBS, a time longer than that used for experiments. The results obtained in irradiation experiments must therefore represent a true synergistic action of TPA on UV irradiated CHO-K1A cells.

TPA enhanced the lethal effects of 313 nm UV to a greater extent than 365 nm UV (Table 6.2). This observation indicates that far from being a transition region, as proposed by Jagger (1985) mid-UV may in fact possess a unique action of cell lethality.

The response of cells *in vitro* to mid - UV has been associated with the release of agents derived from membrane phospholipids. 313 nm UV has been shown to induce the release of prostaglandins (PG) and arachidonic acid (AA) from the membranes of cultured C3H/10T½ mouse fibroblasts (De Leo et al., 1984) and human keratinocytes (De Leo et al., 1985). In these cell systems the release of AA following UV irradiation was rapid and could be inhibited by dexamethasone. As this steroid inhibits the activity of the membrane enzyme phospholipase A_2, this has led to the proposal that mid-UV photons interact directly with cell membranes to activate this enzyme (De Leo et al., 1985). The effects induced in C3H/10T½ cells by 313 nm are similar to those observed following treatment of these cells with phorbol diesters (Mufson et al., 1981), which also specifically activate phospholipase A_2 (Ocuhi and Levine, 1978b; Galey et al., 1985). The enhancement of 313 nm UV-induced inactivation by TPA in CHO-K1A cells may represent the combined action of these two agents at the cell membrane. While the enhancement of 365 nm UV-induced cell lethality probably represents a less specific UV membrane effect together with this specific action of TPA.

Unfortunately, parallel mutagenesis enhancing studies with TPA and UV of specific wavelength were not possible due to technical constraints. The large numbers of cells required for mutagenesis studies cannot be irradiated in suspension using the optical bench arrangement in Fig. 6.1a. Large numbers of cells can be irradiated attached to T/C dishes, but sources of monochromatic light are, as yet, unavailable for this method of irradiation.

The interaction between TPA and UV, particularly at 313 nm, suggests that a clastogenic factor may be induced in CHO-K1A cells by TPA. Therefore, the mutagenesis enhancing activity observed for TPA in the

CHO-K1A/Oua^R system may be wholly due to a particular set of experimental conditions.

The activation of phospholipase A_2 and the subsequent production of a clastogenic factor in cells treated with TPA may be mediated through the specific phorbol diester receptors (Cerutti and Amstad, 1983). In CHO-K1A general membrane perturbations, such as those induced by linear alkanes, do not lead to an enhancement of mutagenesis. Therefore, the enhancing effects of TPA in these cells might be mediated through such a receptor interaction. CHO, and presumably CHO-K1 cells do possess reversible ^{3}HPDBu binding sites (Shoyab et al., 1980). The negative results for TPA in CHO(S) cells (Table 5.5) may, therefore, be simply explained by the mutagen concentration effect, observed in CHO-K1A cells (3.3). The extreme sensitivity of CHO(S) cells to the toxic effects of MNNG precluded further investigation of this concentration effect in these cells.

APPENDIX

The V79 and CHO cell lines maintained for these studies are extremely sensitive to the toxic effects of MNNG (see 5.2.1 and 5.3.1), when compared with the results obtained for similar cells treated under similar conditions by other workers (Kao and Puck, 1968; Roberts et al., 1971; Peterson et al., 1979). Throughout the present studies a single batch of MNNG (Sigma Chemicals Lot no. 47C-0143) was used. It was possible that this may have degraded or become contaminated with other toxic compounds during storage. A fresh batch of MNNG was thus obtained from the same supplier (Sigma Chemicals Lot no. 112F-0003). The purity of these two batches was then compared using a spectrophotometric method and a parallel toxicity study was undertaken using single-plated CHO-K1A cells.

The ultraviolet absorption spectrum for MNNG should exhibit two maxima at 280 nm and 400 nm (Gichner and Veleminsky, 1982). The molar extinction coefficients at these maxima are 1.77×10^{-4} $M^{-1}cm^{-1}$ and 167.9 $M^{-1}cm^{-1}$, respectively (La Polla et al., 1972). Upon decomposition the peak at 400 nm disappears, and the 280 nm peak is replaced by one at 260 nm corresponding to the absorption maxima for N-nitrourea (La Polla et al., 1972). The absorbance at 400 nm is linear with increasing MNNG concentration and is usually used to measure the stability of MNNG.

The purity of the MNNG batches was assessed by the spectrophotmetric method of La Polla et al. (1972). For each batch a solution was prepared in DDH_2O in the usual way at a concentration of 500 µg/ml (see 2.4.1). A portion of each was sterilised by filtration through 0.2 µm pore size Millex-GS filter unit (see 2.2.1), and stored at -20°C

protected from light. This was used in the parallel toxicity study.

Ultraviolet and visible absorption spectra (200 - 600 nm) were measured on a Perkin Elmer 500S automatic spectrophotometer for MNNG solutions in matched, stoppered quartz cuvettes. For both batches, a similar absorption spectrum was observed and the absorption maxima detected for MNNG at final concentrations of 5 µg/ml and 500 µg/ml are given in Figs. A.1a and b. Over the wavelengths scanned (200 - 600 nm) no other absorption maxima were observed. Particularly no peak at 260 nm, indicative of MNNG degradation, was observed for either batch. The absorption spectra obtained are in agreement with that given by La Polla et al. (1972).

Using the molar extinction coefficient at 400 nm the concentration of MNNG can be calculated. For the two batches tested the MNNG concentrations are given in Table A.1.

Table A.1. MNNG concentrations calculated from absorbances at 400 nm for different batches of MNNG.

MNNG batch	Absorption maximum(nm)	Absorbance	MNNG concentration (µg/ml)
47C-0143	398	0.536	469
112F-0003	399	0.554	485

The toxicity of MNNG was examined in single-plated CHO-K1A cells grown in F10 medium following the method used previously (see 3.4.1). The dose-response curves obtained from these studies for each batch of MNNG are given in Figs. A.2a and b. Both curves are superimposable upon that given in Fig. A.2c, obtained previously for CHO-K1A cells

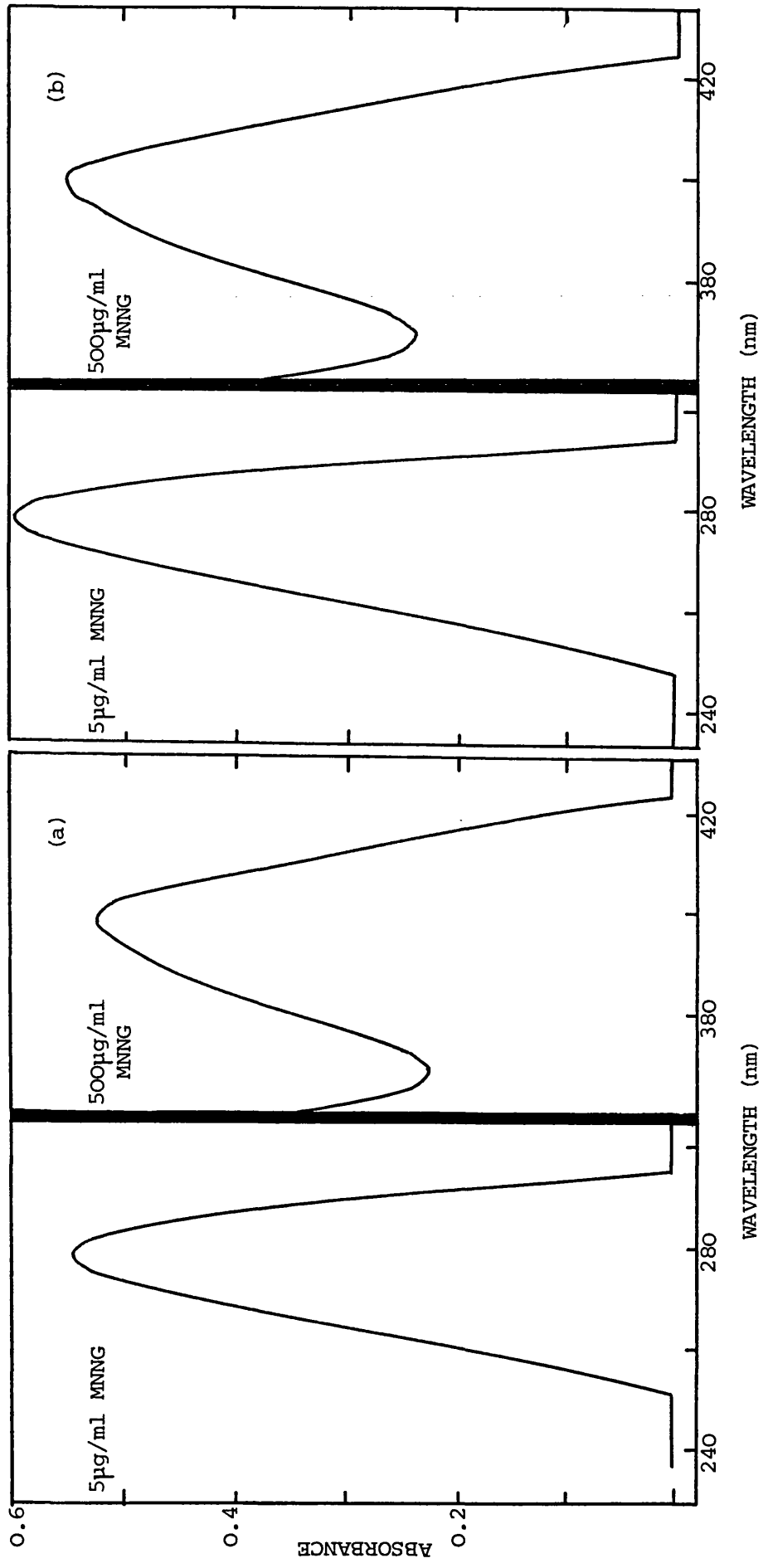


Fig. A.1 UV absorption spectra for two different batches of MNNG (a) Lot No 47C-0143 (b) 112F-0003.

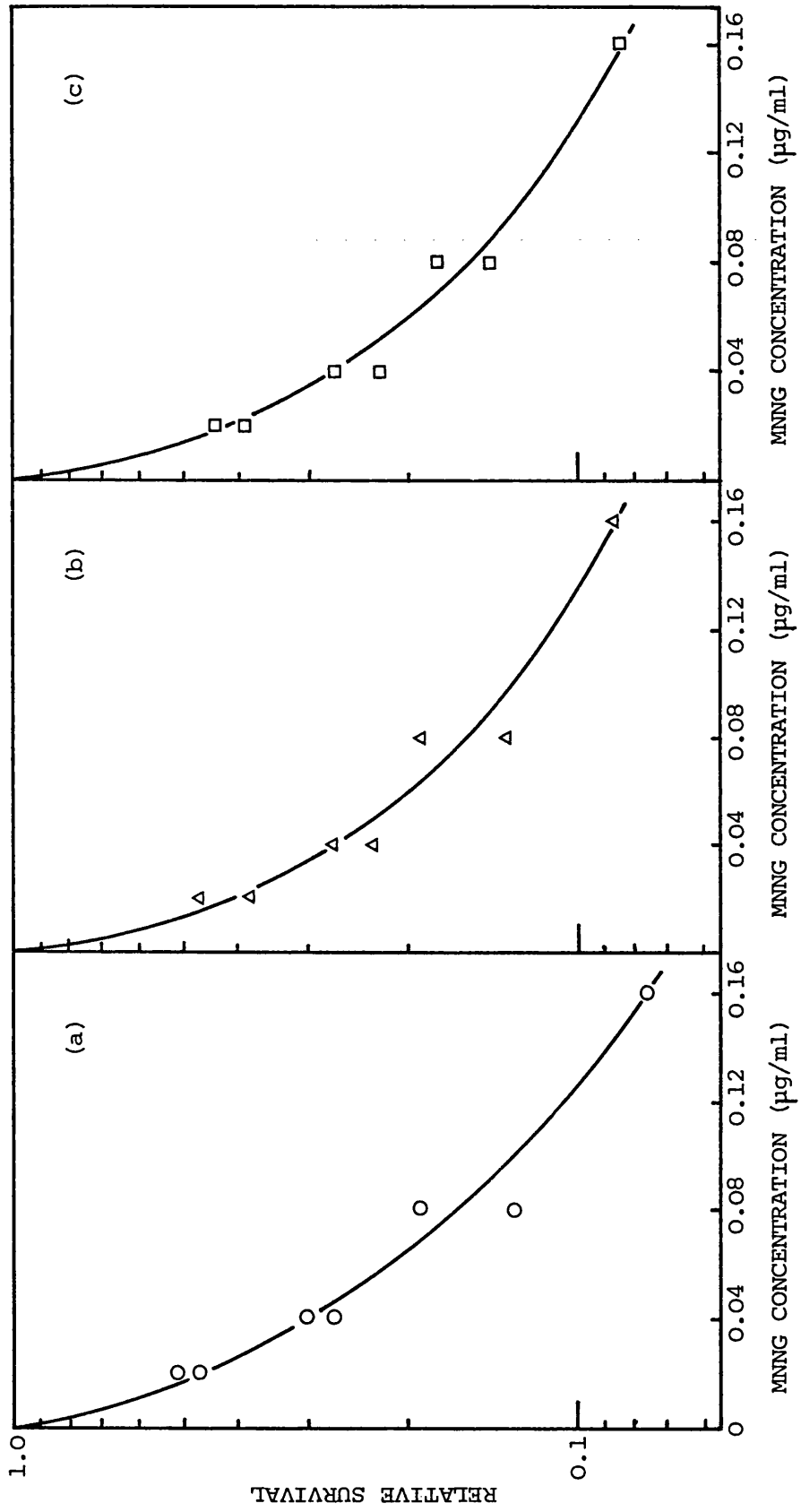


Fig. A.2 Dose-response curves for single-plated CHO-K1A cells grown in F10 treated with MNNG (a) Lot No 47C-0143 (b) Lot No 112F-0003 (c) Lot No 47C-0143 redrawn from Fig. 3.1a.

treated with MNNG (Lot 47C-0143) redrawn from Fig. 3.1a. The D_{37} values are 0.024 μg/ml from Fig. A.2a and 0.022 μg/ml from A.2b. The D_{37} value for single-plated CHO-K1A cells treated with MNNG interpolated from Fig. A.2c is 0.02 μg/ml.

The results from these investigations indicate that there are no obvious differences between the two batches of MNNG examined. The extreme sensitivity of the V79 and CHO cell lines must therefore be due to other factors.

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